

Vol. 78, B, Part II (April to June) 2008

ISSN 0369-8211

Proceedings of the National Academy of Sciences, India

Section B - Biological Sciences



Published by

The National Academy of Sciences, India

5, Lajpatrai Road, Allahabad-211002

PROCEEDINGS OF THE NATIONAL ACADEMY OF SCIENCES, INDIA
(SECTION - B, BIOLOGICAL SCIENCES)

EDITORIAL BOARD

Prof. B.N. Dhawan
Chief Editor

Formerly Director, Central Drug Research Institute, Lucknow,
3, Ram Krishna Marg, Lucknow-226007
Fax : 091-0522-2623405 E-Mail bndhawan@hotmail.com
(Neurosciences/Pharmacology)

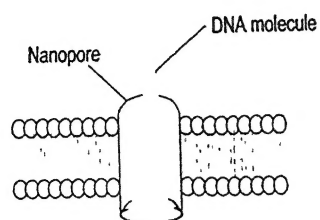
1. Dr.S A.H Abidi
Formerly Member, Agricultural Recruitment Board,
CM 11, Sector B, Aliganj, Street & City Expansion
Scheme, Aliganj, Lucknow- 226020
E-mail sahabidi@hotmail.com
(Marine Biology/Fisheries)
2. Prof. R.S Ambasht
Professor Emeritus & INSA Senior Scientist
Deptt. of Botany, B.H.U ;
97,Jawahar Nagar Extension,
Varanasi- 221010
Fax 091-0542-2368174
E-mail rambasht@banaras.ernet.in
(Ecology and Environmental Sciences)
3. Prof Subhash Chand
Department of Biochemical Engineering & Bio-
technology
Indian Institute of Technology-Delhi
Hauz Khas, New Delhi-11 0016
Fax 091-011-26582282
Email subhashc46@hotmail.com
(Biochemical Engineering)
4. Dr.Madhu Dikshit
Scientist E-II
Division of Pharmacology
Central Drug Research Institute,
P B 173, Lucknow-226 001
Fax 091-0522-2623405,2623939
E-mail madhudikshit@yahoo.com
(Biochemical Pharmacology)
5. Prof.P.K.Gupta
Honorary Emeritus Professor & INSA Senior
Scientist, Molecular Biology Laboratory
Department of Genetics and Plant Breeding
Ch.Charan Singh University, Meerut- 250004
Fax : 091-0121-2768195
E-mail: pkgupta36@vsnl.com; pkgupta36@hotmail.com
(Cytogenetics/Genetics and Plant Breeding/Crop
Biotechnology)
6. Prof. M.S.Jairajpuri
Professor of Zoology
Aligarh Muslim University
Aligarh - 1202002
E-Mail . jairajpurims@lycos.com
(Nematology/Environment/Taxonomy Evolution)
7. Prof.V.P.Sharma
M N Saha Distinguished Fellow, NASI,
Formerly Additional Director-General,
Indian Council of Medical Research &
Director, MRC, Delhi, Res: C5/10(GF),
Vasant Kunj, New Delhi- 110070
Email vinodpsharma@gmail.com
(Entomology/ Malariaology)
8. Prof. C.B.L.Srivastava
Formerly Professor & Head
Department of Zoology,
University of Allahabad,
78 Balrampur House
Allahabad-211002
(Fish Anatomy, Neurobiology)
9. Prof. (Mrs.)Veena Tandon
Dean, School of Life Sciences
North Eastern Hill University
Shillong -793022
Fax- 091-0364-2550300, 2550108
E-mail tandonveena@hotmail.com, tandonveena@gmail.com
(Parasitology-Helminthology)
10. Dr.Rakesh Tuli
Director
National Botanical Research Institute
Rana Pratap Marg,
Lucknow- 226001
Fax 091-0522-2682849, 2682881
E-mail. manager@nbri.resnetd.ernet.in
(Plant Biochemistry/ Molecular Genetics)
11. Prof. H.N.Verma
Rector , Seedling Academy of Design,
Technology and Management,
Seedling Campus, Jagatpura
Jaipur- 302025
E-mail. vermalko@yahoo.co.uk
(Plant Virology & Plant Pathology
Plant Protection/Molecular Biology)
12. Dr.Rishendra Verma
Head, Division of Biological Standardization
Indian Veterinary Research Institute
Izatnagar - 243122
Fax: 091-0581- 230-3284
Email rishendra_verma@yahoo.com
(Microbiology / Immunology/Mycology)

Prof.G.K.Srivastava
Managing Editor

Formerly Professor & Head, Department of Botany, AU, Formerly
Member, U P Higher Education Commission,
10/1 Bank Road, Allahabad - 211002
Email nrajnatacademy@hotmail.com
(Palaeobotany/Morphology/Pteridology/Cytogenetics)

CONTENTS

Proc. Nat. Acad. Sci. India, Sect. B, Vol. 78 Pt. II, 2008

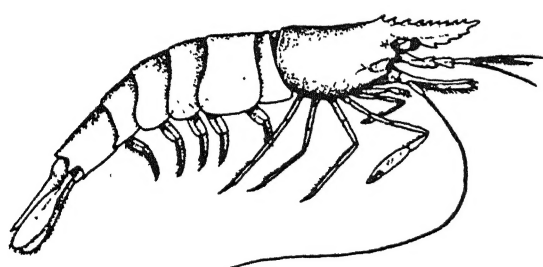


Ultrafast and low-cost DNA sequencing methods for applied genomics research

P.K. Gupta

91-102

The availability of genome sequences of a number of organisms and the need for resequencing of the genomes for the study of genome variation has created a demand for ultrafast low-cost DNA sequencing methods. Consequently, several new generation DNA sequencing platforms have been developed during 2005-2008, which have been briefly described in this review.

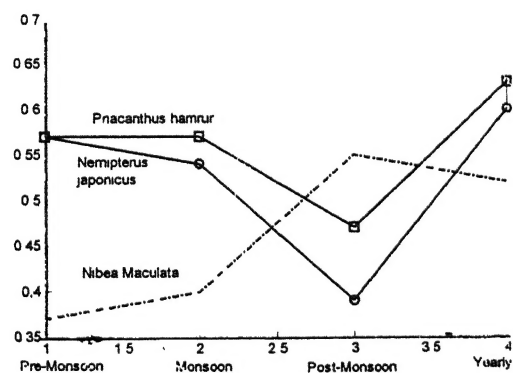


Major diseases and the defence mechanism in giant freshwater prawn, *Macrobrachium rosenbergii* (de Man)

Shailesh Saurabh and P.K. Sahoo

103-121

This review focuses on the recent information of major diseases of giant freshwater prawn, *M. rosenbergii* de Man. and the related defence mechanism which may be of help for sustainable development of fast-growing scampi industry.

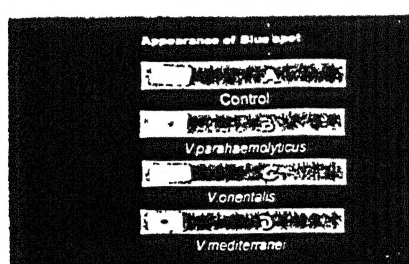


Impact of trawling on resource partitioning among certain demersal fishes of the Kerala coast

Asha Nair M. and C.M. Arvindan

122-132

The present study is undertaken to investigate the change in resource partitioning among three demersal fishes due to trawling viz. *Nemipterus japonicus* (Bloch), *Priacanthus hamrur* (Forsskal) and *Nibea maculata* (Bloch and Schneider) at the Kerala coast. Quantitative analysis of stomach contents, based on several statistical indices including diet overlap, diet breadth and index of relative importance indicate the change in dietary patterns of the fishes. Results of the study revealed interesting diet sharing patterns among the selected demersal fishes at trawled and non-trawled sites.



Detection of *Vibrio* proteins from diseased *Penaeus monodon* (Fab.) by ELISA and Western blotting

N. Chandrakala, G. Sampath Kumar, P. Rebekka, G. Kiruba Jasmine, M. Prabakaran and M. Ayyavoo

133-136

Proteins of *Vibrio* sp. isolated from black spot diseased *P. monodon* were subjected to ELISA and Western blotting. SDS-PAGE analysis showed 29 kDa proteins.

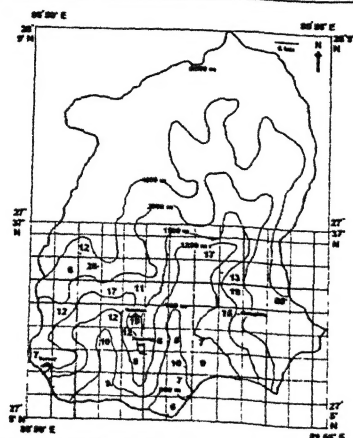


Differential Giemsa staining patterns in chromosomes of three Iranian species of *Fritillaria* spp. group

Gholamreza Bakhshi Khaniki

137-146

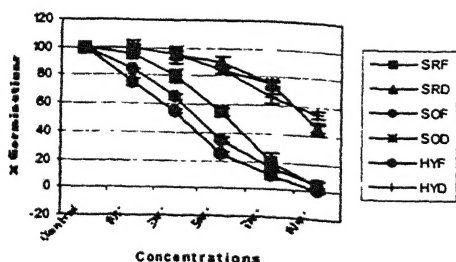
On the basis of C-band patterns and other karyologic features three species are described for the Iranian *Fritillaria* spp. group, *F. caucasica* (Adams), *F. uva-vulpis* (Rix) and *F. assyriaca* (Baker). All the three species have a similar basic karyotype ($n=12$). Further aspects of constitutive heterochromatin, heteromorphism, equilocal C-band distribution, C-banding evolution, and the role of C-banding in taxonomy are discussed.



Studies on the diversity and pattern of vertical distribution of some epiphytic pteridophytes on their host plants of southern Sikkim, India

Gautam Ganguly and Radhanath Mukopadhyay 147-157

Pattern of vertical distribution and diversity of epiphytic pteridophytes on the host trees of Southern Sikkim were studied. The occurrence of epiphytic pteridophytes in relation to the type of forest, altitude and environmental factors have also been determined.



In-situ and ex-situ evaluation of seaweed liquid fertilizers on seed germination, early growth and chlorophyll content of *Trigonella foenum graecum* Linn.

Vaibhav A. Mantri and B.B. Chaugule

158-163

The effects of seaweed liquid fertilizers (SLFs) prepared from *Sargassum tenerrimum* J. Ag., *Hypnea valentiae* Turn and *Solieria robusta* (Grev.) Kylin on seed germination, early growth and chlorophyll content of *Trigonella foenum graecum* Linn. were evaluated *in-situ* and *ex-situ*.

Detection of trichothecenes and other mycotoxins produced by *Fusarium*

Name of the toxin	Solvent system	Spray reagent	Detection	
			UV	Visible
Deoxynivalenol (DON)	C M (97.3)	4,7,8	-, ch, bl	Y, -
Diacetoxyscirpenol (DAS)	C M (97.3)	6, 9	bg, -	-, br
Fusarenone - X	C M (97.3)	8	bl	-
HT-2 toxin	C M (97.3)	6	bg	-
Moniliformin	C M (97.3)	2, 10	-	br, br
Nivalenol (NIV)	C M (97.3)	4, 7, 8	-, chl, bl	Y, -
1-2 Toxin	C M (97.3)	6, 9	bg, -	-, P
Zearalenone	C M (97.3)	1, 2, 3, 5, 7, 8	-, -, br, ch, bl	br, do, lp, -, -
Fumonisin (FB1, FB2)	W M (3.1)	4, 11	-	br

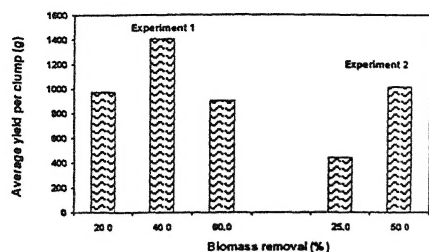
Solvents C = Chloroform, M = Methanol, W = Water

Natural incidence of fusarial mycotoxins

K. Narasimha Rao, B. Vijayapal Reddy, S. Girisham and S.M. Reddy

164-168

An extensive and intensive survey of foods (maize and sorghum) of different regions of AP was carried out for the presence of different fusarial species and their toxins. Fumonisin, zearalenone, T₂ toxin, DON and DAS were some of the toxins detected.



Promotion and cultivation of *Thysanolaena maxima* (Roxb.) Kuntze: A multiuse species in Uttarakhand

Anwesha Sah and Uma T. Palini

169-173

This multiuse species, *T. maxima*, has potential of providing supplemental income to the womenfolk of Uttarakhand besides reducing their workload in terms of fodder collection. The yield obtained at 40% and 60% level of harvest indicates that if broom grass is grown in one hectare of land, close to a village, it can save rural women up to 290 headloads of fodder collection per person in a year.

Environmentally affected allelopathic response of few weeds on late blight disease of potato

S.N. Phukan

174-178

The course of development of late Blight disease of potato was studied in experimental plots planted with seed tubers of cultivar *Khasigaro*, treated with extracts of weed flora namely, *Ludwigia purviflora* Roxb. *Polygonum plabejum* R. Br. and *Anisomeles ovata* Br. The incidence of late Blight was found to be significantly delayed and percent Blight intensity sufficiently decreased when the plants were treated with extracts of *Ludwigia* and *Anisomeles*.

No. of tubers/plant		No. of tubers/plant (average)		
Treatment	No. of plants/plot	2004-05	2005-06	2004-05
Control	118	10.64	12.33	32.76
T ₁	116	15.43	18.87	74.06
T ₂	115	07.52	19.12	24.45
T ₃	112	14.87	24.53	73.76

POSTAL ADDRESS

The National Academy of Sciences, India
5, Lajpatrai Road, Allahabad - 211 002, India

TELEGRAPHIC ADDRESS

NATACADEMY

Phone : +91-532-2640224

FAX

091-0532-2641183

E-MAIL

nasi@sancharnet.in

WEBSITE

www.nasi.org.in

Published by Prof. P.K. Seth, General Secretary for the National Academy of Sciences, India, 5, Lajpatrai Road,
Allahabad-211002 and Printed by Apex Graphics, Allahabad.

Managing Editor : Prof. G.K. Srivastava

Co-sponsored by C.S.T., U.P., Lucknow.

PROCEEDINGS
OF THE
NATIONAL ACADEMY OF SCIENCES, INDIA
2008

VOL LXXVIII

SECTION-B

PART II

Ultrafast and low-cost DNA sequencing methods for applied genomics research

P.K. GUPTA

Molecular Biology laboratory, Department of Genetics and Plant Breeding, CCS University, Meerut, India.

e-mail : pkgupta36@gmail.com

Received September 10, 2007, Accepted November 13, 2007

Abstract

The availability of human genome sequence and the emergence of personal genomics era recently created a demand to bring down the cost of human genome resequencing to US \$ 1000, to make it clinically useful. As a result, sequencing systems using Sanger's technology have been scaled-up, and several ultrafast and low-cost sequencing systems using newer technologies have been developed and launched during 2005-07. Three sequencing platforms, which became available for genomics research in 2007 include the following: (i) Genome Sequencer 20 (launched in 2005) and upgraded Genome Sequencer FLX (launched in 2007), both developed and commercialized jointly by 454 Life Sciences and Roche Applied Science; (ii) Solexa Genome Analyzer (earlier called Solexa 1G) developed by Solexa (later acquired by Illumina) and launched in early 2007, and (iii) SOLiD system, developed and launched in October, 2007 by Applied Biosystems. According to available reports, as many as more than 60 454/Roche Genome Sequencers 20/FLX and at least two dozen Solexa Genome Analyzers are already being used by different genome research centres globally. ABI SOLiD machines will also become available shortly at several centres. Recently, Solexa's sequencing system has also been used for chromatin immunoprecipitation sequencing approach (ChIPSeq) to study DNA methylation and histone modifications at the whole genome level. Several other technologies are also being tested to allow further reduction in cost and time needed for DNA sequencing. For instance, several single molecule sequencing technologies, including nanopore sequencing, which are likely to become available in foreseeable future, should reduce further the cost and time of DNA sequencing. A major

सारांश

कुछ समय पहले तक, मानव जीनोम क्रमबद्धता तथा व्यक्तिगत जीनोम के अध्ययन के अविर्भाव ने इस बात की आवश्यकता पर बल दिया है कि मानव जीनोम क्रमबद्धता अध्ययन की लागत घटकर 1000 US \$ तक आ जाये तो रोगियों को समुचित लाभ मिल सकेगा। परिणामस्वरूप ऐसे जीनोम को क्रमबद्ध करने की व्यवस्थाये विकसित हो गयी है, जिनमें सेगर की तकनीकी का प्रयोग हुआ हो तथा इन्हे 2005 से 2007 के मध्य बाजार में उतार दिया गया है। सन् 2007 तक जीनोम से सबद्ध अनुसंधान हेतु क्रमबद्ध करने की तीन व्यवस्थाये विकसित हो गई है (i) जीनोम सीक्वेन्स 20 (जो 2005 में प्रारम्भ हुआ) तथा उन्नत जीनोम सीक्वेन्सर एफ०एल०एक्स० (जो 2007 में प्रारम्भ हुआ), इन दोनों को एक साथ विकसित करके व्यापारिक व्यवस्था में 454 लाइफ साइसेज तथा रोश अफ्लाइड साइस द्वारा लाया गया। (ii) सोलेक्स जीनोम एनालाइजर (जिसे प्रारम्भ में सोलेक्स आई०जी० नाम दिया गया) सोलेक्स (जो बाद में इल्यूमिना द्वारा अधिग्रहीत कर ली गई) द्वारा विकसित कर के 2007 में प्रारम्भ किया गया। (iii) SOLiD तंत्र जिसे एफ्लाइड बायोसिस्टम द्वारा विकसित करके अक्टूबर 2007 में प्रारम्भ किया गया। प्राप्त सूचनाओं के आधार पर संपूर्ण विश्व के विभिन्न जीनोम अनुसंधान केन्द्रों पर 60454 रोश जीनोम सीक्वेन्सर 20/एफ०एल०एक्स० और कम से कम दो दर्जन सोलेक्स जीनोम एनालाइजर उपयोग में लाये जा रहे हैं। कुछ समय पहले से सोलेक्स सीक्वेन्सिंग तंत्र का भी प्रयोग संपूर्ण जीनोम के डी०एन०ए० मीथाइलेशन तथा हिस्टोन रूपान्तरण के अध्ययन में क्रोमैटिन इम्पूनोप्रेसिपिटेशन क्रमबद्धता विधि (ChIPSeq) द्वारा किया जा रहा है। डी०एन०ए० की क्रमबद्धता के अध्ययन में, लागत तथा समय बचाने के उद्देश्य से और भी दूसरी तकनीकी विधिया प्रयोग में लाई जा रही हैं। उदाहरणार्थ, अनेक एकल परमाणु क्रमबद्धता विधिया जिनमें नैनोपोर क्रमबद्धता विधि भी शामिल है, पूर्वानुमानित भविष्य में प्रयोग में लाई जा सकेंगी जिससे

limitation with some of these modern sequencing systems (e.g. Solexa and ABI systems), however, is the upper limit of read lengths of about 35bp per run. But, in parallel with the development of these sequencing systems, computer algorithms are being developed, which will allow assembly of these small sequences into whole genome sequence, to allow de novo whole genome sequencing even with these machines providing only short read lengths. All these aspects involving a revolution in DNA sequencing technologies are briefly discussed in this brief review.

Key words : human genome, genome sequencer 20, solexa genome analyzer, SOLiD system, ChIPSeq, DNA methylation, histone modification, nanopore sequencing.

Introduction

During the current genomics era that started in mid-1990s, whole genome sequencing projects have been completed for a large number of organisms, including several hundred microbes and more than a dozen eukaryotes (including both animal and plant systems). Among these projects, the Human Genome Project (HGP) that was successfully executed and completed in April 2003 was perhaps the biggest biology project (costing US\$ 2.7 billion of FY 1991)¹. The HGP project was compared by some with projects, which involved either the man's landing on moon or the splitting of atom. The completion of these whole genome sequencing projects including HGP created a demand for large-scale high-throughput DNA sequencing, which has never been greater in the past. Although the cost and time for large-scale DNA sequencing is declining steadily, the common sequencing technologies in 2004 were still too expensive and time-consuming to be of practical utility, either in agriculture or in human health-care. Keeping this in view, National Human Genome Research Institute (NHGRI) in 2004, initiated a project entitled "Revolutionary Genome Sequencing Technologies: The \$1,000 Genome", aiming at bringing down the cost of whole genome resequencing in humans (for detection of DNA polymorphisms like SNPs) initially to \$ 100,000, and eventually to US \$ 1000, so that it may become clinically useful for the so-called personal genome project (PGP) during the current era of personal genomics² (PGP envisages integration of data from genomes, environments and phenotypes from more than 100,000 human volunteers). Following this announcement, rapid progress has been made in the development of new cost-effective and time-saving DNA sequencing technologies. The

डीएनए की क्रमबद्धता के अध्ययन की लागत तथा समय घटाया जा सकेगा। इन आधुनिक क्रमबद्धता के अध्ययन की व्यवस्थाओं में कुछ एक (सोलेक्सा और सीबीआई) व्यवस्था में, एक बड़ा परिसीमन है इनकी "रीड लेन्थ" की ऊपरी सीमा का लगभग 35 बीपी प्रति रन का होना। परन्तु इन सीक्वेन्सिंग व्यवस्थाओं के विकास के साथ ही साथ कम्प्यूटर एल्गोरिथ्म का भी विकास हो रहा है, जिससे इन छोटे छोटे क्रमों को मिला कर पूरे जीनोम सीक्वेंस का विस्तृत अध्ययन किया जा सकेगा। इस प्रकार ऐसी मशीनों के द्वारा भी जिनमें केवल छोटे छोटे "रीड लेन्थ" मिलते हों, पूरी जीनोम क्रमबद्धता का अध्ययन किया जा सकता है। प्रस्तुत पुनरावलोकन में डीएनए क्रमबद्धता प्रौद्योगिकी क्रांति से संबंधित सभी प्रारूप वर्णित किये गये हैं।

सांकेतिक शब्द : मानव जीनोम, जीनोम सीक्वेन्सर 20, सोलेक्सा जीनोम एनालाइजर, SOLiD तंत्र, ChIPSeq, डीएनए मिथाइलेशन, हिस्टोन रूपान्तरण, नैनोपोर क्रमबद्धता।

published literature and the progress made in this connection have been reviewed in the recent past^{3,4}, and it has been shown that the progress made actually outpaced earlier expectations⁵.

In the early years of the present century, it was recognized that the reduction in the cost and time of sequencing will depend mainly on the following technological developments: (i) miniaturization in instrumentation, (ii) microfluidic separation of substances or molecules, (iii) increase in the number of assays per run, and (iv) development of novel detection methods. These developments sometimes also led to the development of 'lab-on-a-chip' systems (see. Special Issue, Nature Insight, July, 2007). It should be emphasized, however, that we will need new sequencing strategies to make use of these high-throughput platforms effectively. The new strategies leading to the development of platforms for ultrafast low-cost DNA sequencing that have either been developed or are currently under development fall under the following four categories; (i) sequencing-by hybridization (SBH), (ii) sequencing by synthesis (SBS), (iii) sequencing by ligation, and (iv) nanopore sequencing. The present article gives a brief overview of the development of these new DNA sequencing strategies.

Sequencing by hybridization (SBH)

Sequencing by hybridization (SBH) is based on the principle that differential hybridization of oligonucleotide probes, each due to mismatch of a single base, can be used to decode the target DNA sequence. There are at least two alternative approaches for SBH; (i) In the first approach, genomic DNA to be sequenced is first immobilized on a membrane or a glass plate, and is then serially hybridized with short oligonucleotide

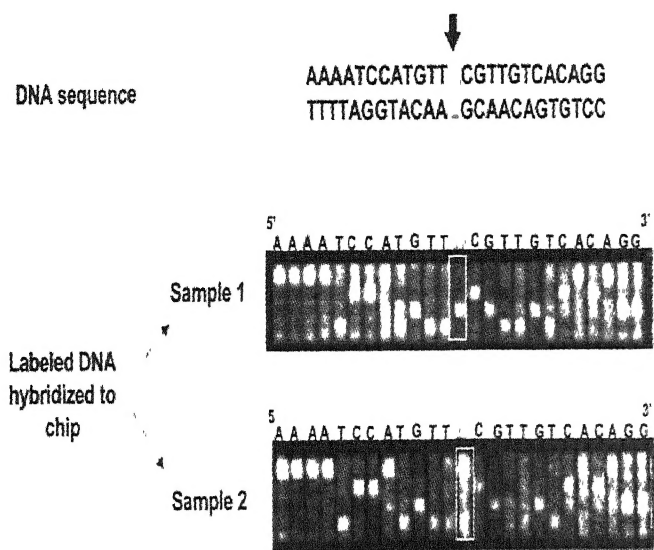


Fig. 1 - A diagrammatic representation of the technique of sequence by hybridization (SBH) using a hypothetical example of single nucleotide polymorphism (modified with permission from Perlegen Sciences). A sequence of 25 base pairs is shown at the top with an arrow at 13th base pair, which is the site of SNP. Two panels of hybridized chips show hybridization patterns with two samples, which differ for an SNP at 13th position (GC—> AT).

probes (~7bp) of known sequences. The level of hybridization with each of millions of probes is used to infer the genome sequence. The approach has already been used both for resequencing and de novo sequencing^{6,7}. (ii) In the second approach, genomic DNA to be sequenced is hybridized to microfabricated tiling arrays of immobilized oligonucleotides, with ~100,000 copies of each individual oligonucleotide feature (~25bp long) per μm^2 . For each base pair of a genome to be sequenced, there are four features available on the chip corresponding to four possible bases (A, C, G, T). The number of features per base pair will become eight, if both strands are to be examined (as done recently by Perlegen in designing rice oligonucleotide arrays). The strongest signal with only one of the four or with two of the eight features in each case will enable resequencing or sequencing of the whole genome or a part of it. The principle involved in SBH is discussed in more detail elsewhere⁸, and is illustrated in Figure 1. The method is more frequently used for detecting single nucleotide polymorphisms (SNPs), and has been successfully used by Perlegen for initially resequencing human chromosome 21⁹, and is currently being used by them for resequencing the rice genome for detection of SNPs.

Sequencing by synthesis (SBS)

The SBS technology encompasses many different DNA polymerase-dependent strategies, so that the term SBS has become increasingly ambiguous; therefore, the following classification of DNA polymerase-dependent strategies may prove useful: (a) *sequencing by Sanger's method*, (b) *sequencing by 'single nucleotide addition' (SNA)*, (c) *sequencing by 'cyclic reversible termination' (CRT)*, (d) FISSEQ polony sequencing, and (e) cyclic array single molecule sequencing.

Sequencing by Sanger's method: current DNA sequencing platforms

Till recently, DNA sequencing techniques involved collection of DNA from an organism (including humans) and then making billions of copies of it to run through sequencing machines. This is slow, expensive, and error prone. This DNA sequencing method (also used for Human Genome Project) was the result of advances in several technologies including the following: (i) enzymology, (ii) fluorescent dyes, (iii) dynamic coating polymers and their derivatives, (iv) capillary array electrophoresis (CAE), and (v) fluorescence detection. In automatic sequence analysers based on these technologies, either the primers or the ddNTPs are labeled with specific fluorescent dyes facilitating detection of fragments resulting from termination of DNA synthesis due to incorporation of ddNTPs.

Improvement over CAE and four colours dye-labeled nucleotides.

The two high-throughput Sanger's method-based CAE automated DNA sequencing machines, which have been extensively used in the past include the following: (i) Applied Biosystems 3730 DNA Analyzer (a 96-capillary DNA sequencer and genotyper); and (ii) the GE MegaBACE DNA Analysis System. The MegaBACE 4000 DNA Analysis system allows sequencing labs to generate up to 2.8 million bases in a 24-hour period by processing 384 samples simultaneously. In recent years, improvements have been made in the above two earlier machines both in respect of technology used for separation of DNA fragments and also in respect of technology used for the detection of separated molecules (see below).

(i) *Microelectrophoretic/microfluidic separation*. Several teams, including researchers at the BioMicroElectroMechanical Systems (BioMEMS) laboratory (located at the MIT Whitehead Institute for

Biomedical Research, Cambridge, Mass.) investigated if costs can be reduced by additional multiplexing and miniaturization of the earlier systems. For this purpose, electrophoresis was performed on microfabricated plates, which represents a very practical, near-term advance using standard Sanger methodology. This led to the production of BioMEMS 768, which was designed specifically for *de novo* long-read sequencing and uses a large format, 384-lane plate (Figure 2). With two of these plates used together, the setup has 768 lanes¹⁰. In terms of lane count, BioMEMS 768 is eight times the capacity of the Applied Biosystems 3730 DNA Analyzer or twice that of the GE MegaBACE DNA Analysis System.

Although the separation principles of microfabricated devices are similar to those of CAE, but the injection

methods differ. For CAE, the sample is introduced by electrokinetic injection into the capillary, but in microfluidic devices, the sample is introduced via a channel network by a variety of process strategies. Thus the time spent in sample injection and in separation of DNA fragments is substantially reduced.

(ii) *Fluorescence detection (FRET and PME technologies)*. The most commonly used detection system in sequencing machines that are based on Sanger's method involved four colours, one each for four different nucleotides, which were dye-labeled. These four-colour systems had several disadvantages includ-

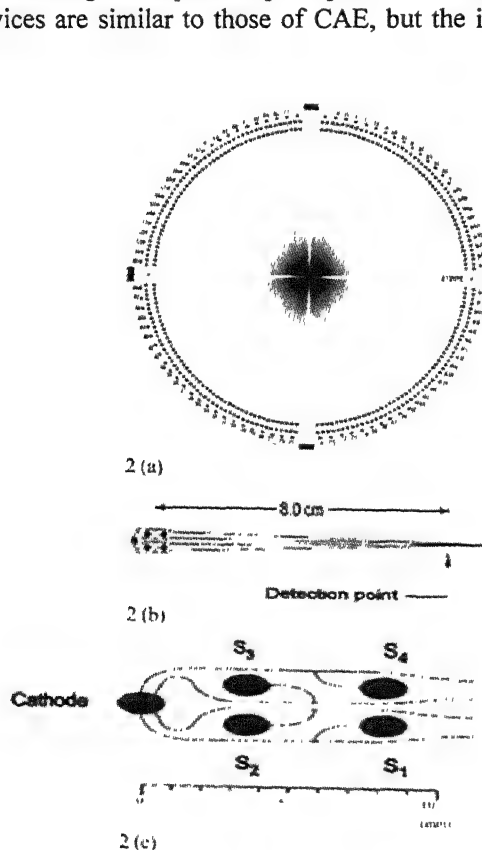


Fig. 2 - A representation of miniaturized microfabricated device for high throughput multiplexing in Sanger's method of DNA sequencing: (a) layout of the 384-lane CAE (capillary array electrophoresis) device on a 200-mm-diameter wafer; (b) expanded view of a single quartet of channels with their injectors; (c) expanded view of one end of a quartet of channels showing a common cathode reservoir located closest to the edge of the wafer (reproduced with permission from Analytical Chemistry, Oct., 2002, 74, page 5077).

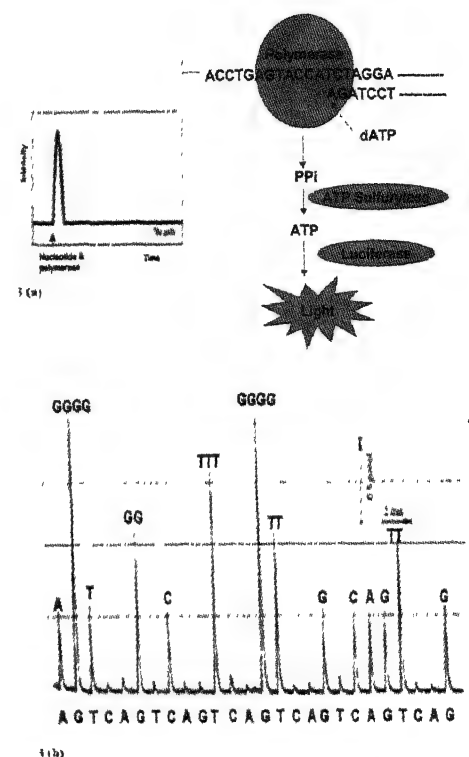


Fig. 3 - Principle involved in DNA sequencing through pyrosequencing used by 454 Life Sciences: (a) schematic representation of the progress of enzyme reaction in solid-phase pyrosequencing. The four different nucleotides are added stepwise to the immobilized primed DNA template and the incorporation event is followed using the enzyme ATP sulfurylase and luciferase. After each nucleotide addition, a washing step is performed to allow iterative addition; (b) a pyrogram of the raw data obtained from liquid-phase pyrosequencing (proportional signals are obtained for one, two, three, and four base incorporations; nucleotide addition, according to the order of nucleotides, is indicated below the pyrogram and the obtained sequence is indicated above the program).

ing the following: (i) inefficient excitation of fluorescent dyes; (ii) significant spectrum overlap, and (iii) inefficient collection of emission signals. The problem of inefficient excitation was largely overcome through the use of fluorescence resonance energy-transfer (FRET) dyes^{11, 12}. FRET-labeled ddNTPs are largely used for DNA sequencing now, although signal intensities are suboptimal, when compared with those of single dyes that are excited at their absorption maxima by the appropriate laser source (see below).

A simple but effective method termed 'pulse multiline excitation' (PME) was also described for multifluorescence discrimination¹³. Advantages of PME include the following: (i) absorption maxima for the four fluorescent dyes are matched to the excitation sources producing maximum signal intensities; (ii) temporal separation of the laser pulses and expansion of the dye set across the visible spectrum eliminate cross-talk between the dyes; and (iii) collection of emission signal is improved by eliminating the requirement for dispersion devices (prism, etc.) for colour separation. In other words, PME measures multicomponent assays in a colour blind manner.

Improvements in the technologies as above have made it possible to sequence one mammalian size genome in one month. This is translated into finishing the draft sequence of a mammalian genome in six months (since six times the genome needs to be sequenced) at a cost of US \$ 12 million. Significant advances (discussed later in this article) are needed to bring it to the level of getting complete human genome sequenced within a few hours at an initial cost of \$ 100,000, and to an ultimate cost of \$ 1000.

Sequencing by single nucleotide addition (SNA): pyrosequencing (454/Roche System)

'Single nucleotide addition (SNA)' methods such as pyrosequencing use limiting amounts of individual natural dNTPs to cause DNA synthesis to pause (whenever the limiting nucleotide is required for extension). Unlike Sanger's method, synthesis can be resumed with the addition of the complementary nucleotide. A sequential addition of dNTPs and the generation of chemiluminescence signal (through luciferin) that is proportional to the number of molecules of the same base added, is used to get the sequence (Figure 3). The amount of a given dNTP needs to be limited to minimize misincorporation effects observed at higher concentrations. A major drawback with this

SNA approach is the incomplete extension through homopolymer repeats.

An emulsion method for DNA amplification and an instrument for sequencing by synthesis using a pyrosequencing protocol involving solid support and picolitre-scale volumes was initially optimized by 454 Life Sciences (Branford, Connecticut). This gave 100-fold increase in throughput over current Sanger sequencing technology¹⁴. Later, 454 Life Sciences and Roche Applied Science jointly commercialized sequencing systems based on emulsion method of single molecule DNA amplification and pyrosequencing. In this system called 'Genome Sequencer 20 System', no gels or fluorescent molecules are needed and hundreds of thousands of DNA fragments can be analysed in parallel, making it suitable for whole genome sequencing or genome-wide SNP genotyping. An upgraded Genome Sequencer FLX system, later launched, allows more sequencing cycles and therefore, longer reads than the Genome Sequencer 20 earlier launched. The 454/Roche pyrosequencing technology has already been successfully used for sequencing of the genome of a Neanderthal reported in 2003¹⁵, and that of the genome of James Watson reported in May, 2007. In October 2007, as many as 60 454/Roche sequencing systems were already being used at Genome centres world-wide², and further improvement in these machines are being tried.

Following steps are involved in the technology used in 454/Roche Sequencing System;

(i) *DNA library preparation*: A single-stranded DNA (sstDNA) library with DNA fragments incorporating primer binding adaptors is first prepared using a series of enzymatic steps. For this purpose, genomic DNA (3µg) is first fragmented by nebulization into smaller fragments (300-800bp), which are polished (blunted) before short adaptors A and B are ligated, and a streptavidin binding site is created in each fragment for sample purification. Single DNA molecules are isolated by limiting dilution. The sstDNA library produced thus is assessed for quality and subjected to titration for finding out the optimal amount of DNA (copies per bead) needed for emPCR (em = emulsified).

(ii) *emPCR amplification*. The sst library prepared as above is immobilized onto specially designed DNA Capture Beads, each bead carrying a single fragment. The bead-bound library is emulsified with amplification reagents in a water-in-oil mixture. Each bead is separately captured within its own microreactor

for PCR amplification, which is performed in bulk thus producing clonally amplified library on beads, each bead carrying millions of copies of a specific fragment to be used for sequencing. Individual fragments on their own beads are clonally amplified within the droplets of an emulsion.

(iii) *Preparation of PicoTitre Plate device.* After PCR amplification, a combination of amplified sst library on beads and sequencing enzymes are loaded onto hundreds of thousands of wells available in PicoTitre Plate device, which is then placed into the instrument for sequencing-by-synthesis (GS20/FLX Sequencing System).

(iv) *Sequencing-by-synthesis.* Sequencing starts with a flow of sequencing reagents (buffers + nucleotides) across wells of the PicoTitre Plate. Each sequencing cycle involves flowing of individual nucleotides in a fixed order (e.g., T, A, C, G), so that if the nucleotide is complementary to the available base in the template, the strand is extended releasing a pyrophosphate molecule, which produces a chemiluminescent signal with luciferin (signal strength is proportional to the number of nucleotides incorporated in a single flow). Instead, if the supplied base is not complementary, there is a pause, and no signal is produced. These cycles of SNA proceed concurrently in all wells of the PicoTitre Plate, which permits DNA sequencing of hundreds of thousands of DNA fragments in parallel.

Sequencing by 'cyclic reversible termination' (CRT): Solexa technology

This CRT method used in Solexa technology makes use of nucleotides that are reversibly fluorescent-labeled and also protected against any condensation reaction for further SNA, unless deprotected again. Consequently, after each single nucleotide addition, no further extension is possible unless the terminal nucleotide is first deprotected. The fluorescent label is also erased, so that it does not interfere with the detection of the next nucleotide to be added. Thus each cycle of SNA involves addition of single nucleotide, its detection using a sensitive device, erasure of fluorescence and deprotection of the terminal nucleotide, followed by addition of the next reversibly labeled and protected nucleotide (Figure 4). Solexa has standardized this technology and has developed a sequencing system that was marketed in 2007. The system developed by Solexa allows a single person to produce up to 40 megabases of sequencing in a

4.5 hour run. Sequencing a new organism thus takes approximately one week from start to finish, with repeat runs taking as little as one day to complete. During 2006, Solexa demonstrated an yield of 1G (1G = 1 gigabase = 10^9 bases) high-quality DNA sequence data per flow cell after filtering, using multiple runs on multiple instruments, making Solexa Genome Analysis System the first to achieve this milestone. Solexa Sequencing System was initially called 'Solexa 1G', but later named 'Genome Analyzer'. Solexa's Genome Analyzer has already been used for the study of the patterns of chromatin structure through chromatin immunoprecipitation sequencing (ChIPSeq) technique, and will certainly be used in future for transcription profiling, microRNA/small RNA discovery, and to identify polymorphisms in closely related genomes (for ChIPSeq, see later in this article). Further improvements will be made in the technology during 2008 and thereafter, so that it will be able to achieve the targeted goal of \$1,000 human genome sequencing within the next 2-3 years.

Following steps are involved in the above CRT technology of Solexa :

(i) *Preparation of full diversity libraries of whole genome:* Whole genome DNA is randomly fragmented and end repaired. Solexa oligonucleotide adapters are ligated on both the ends of each of these fragments. This gives a fully representative genomic library of DNA templates without cloning.

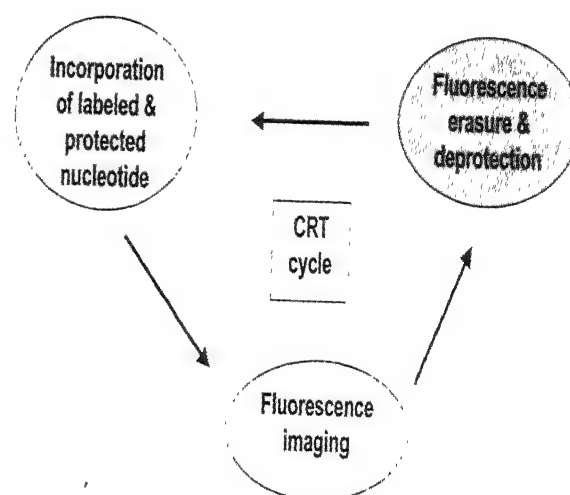


Fig. 4 - A diagrammatic representation of three major events involved in cyclic reverse termination (CRT) during DNA sequencing approach utilized by Solexa.

(ii) *Generating a "Clonal Single Molecule ArrayTM" Flow Cell:* Single molecules from the genomic library are amplified, which involves following six steps: (i) template hybridization; (ii) template amplification; (iii) linearization; (iv) blocking 3' ends; (v) denaturation, and (vi) primer hybridization. These steps are performed on a fully automated Solexa Cluster Station, creating up to 1000 copies of each single molecule, and giving a density of up to 10 million clonal clusters per cm².

(iii) *Sequencing by synthesis.* The flow cell with clonal clusters (each cluster representing only one molecule) is loaded onto the 'Solexa 1G Genetic Analyzer' (later named 'Genome Analyzer'), which conducts automated sequencing by synthesis (SBS). The SBS utilizes four proprietary nucleotides with reversible fluorophores and termination properties. Each sequencing cycle occurs in the presence of all four nucleotides leading to higher accuracy. After incorporation of a nucleotide, reaction terminates, the fluorescence is read and then erased, the terminal nucleotide is deprotected and the new synthesis cycle starts. At each replication cycle, the data is automatically recorded from each cluster, processed and analysed with minimum intervention by the user. This stepwise base addition approach, which cycles between coupling and deprotection, mimics many of the steps involved in automated DNA synthesizers used for synthesis of oligonucleotides.

Fluorescent in situ sequencing (FISSEQ) or bead-based polony sequencing

FISSEQ involves localized amplification of single DNA molecules, using an acrylamide gel, thus creating colonies of PCR product that are called polonies. Because acrylamide restricts the diffusion of DNA, each single molecule included in the reaction produces a spatially distinct micrometer-scale colony of DNA (a polony), which can be sequenced *in situ* independently of similar other polonies (Figure 5).

An extension of the original FISSEQ technique, termed 'bead-based polony sequencing', was developed by Jay Shendure and colleagues in George Church's Lab at Harvard Medical School, Boston, and has been licensed to Agencourt Biosciences, Beverly, Mass. In this massively parallel sequencing-by-synthesis approach, short fragment DNA libraries are clonally amplified onto 1- μ m beads and embedded into a polymer matrix on the surface of microscope slides. The polony slides are then placed into an automated flow cell, where four-

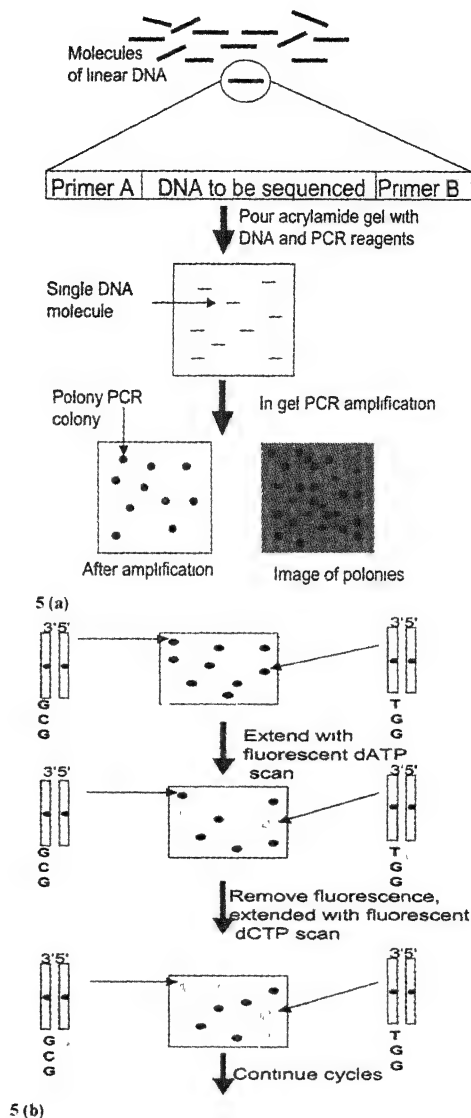


Fig. 5 - (a) Polony amplification. A library of linear DNA molecules with universal priming sites is PCR amplified in a polyacrylamide gel. A single template molecule gives rise to a polymerase colony or polony, (b) Fluorescent in situ sequencing. Polonies are denatured, and a sequencing primer is annealed. Polonies are sequenced by serial additions of a single fluorescent nucleotide.

The sequence GCG shown on the right belongs to the upper right polony and the sequence TGG shown on the left belongs to middle left polony (both sequences shown along with primers and corresponding polonies are marked with arrows). When fluorescent labeled dATP is added in the first round of sequencing reaction, no primer extension is possible in the polony on the right, but extension is allowed in polony on the left; similarly, when dCTP is added in the second round, primer extension takes place in both polonies, thus permitting detection of sequence polymorphism.

colour, fluorescently labeled reagents (corresponding to the DNA bases, A, C, G, or T) are serially delivered to DNA strands. Each polony slide can contain up to 600 million beads with the instrument capable of sequencing 100,000 bases per second, equivalent to approximately 5,000 Applied Biosystems (ABI) 3730 DNA analyzers. It is hoped that the technology will prove useful for personalized medicine and for low-cost genome analysis.

Cyclic array single molecule sequencing

In each of the SNA methods described above, separated single DNA molecules are first amplified, before these are used for DNA sequencing, so that the required signal is achieved. However, if single molecules could be sequenced directly without the need of amplification, this would save cost and time needed for cloning/amplification. Several groups including Solexa, Genovox, Nanofluidics and Helicos are developing cyclic array methods, which though make use of technologies used for other DNA sequencing methods discussed above, but dispense with the amplification of single molecules. Each of these methods makes use of either the excision of labeled mononucleotides using exonuclease I or the extension of DNA fragments by DNA polymerase using fluorescently labeled nucleotides¹⁶. The methods, however, differ in biochemistry and signal detection devices at a resolution of single molecules.

Common approaches for the study of single molecules include fluorescence correlation spectroscopy (PCS) and direct observation of sparse molecules using diffraction-limited optics. These approaches provide observation volumes on the order of 0.20 fl (fl = femtolitre; 1 fl = 10^{-15} litre) and therefore require pico- to nanomolar concentrations of fluorophores in order to isolate individual molecules in solution. However, many biological processes occur at micromolar concentration (rather than nano- or picomolar concentration), so that observation volume has to be reduced by three orders of magnitude to zeptolitre (1 zeptolitre = 10^{-21} litre) in order to isolate single molecules at these higher micromolar concentrations. Zero-mode waveguides consisting of tiny holes (subwavelength diameter = nanopores) in a metal film have been used for studying single molecule dynamics at micromolar concentrations with microsecond temporal resolution¹⁷. Following are some of the methods, where sequencing is achieved using single molecules.

(i) *Reversible terminators*. As discussed earlier, reversible terminators that are being used by Solexa/Illumina sequencing systems, are also being tried for single molecule sequencing by Solexa and Genovox, except that no amplification step will be involved.

(ii) *FRET for serial single base extension*. Quake group makes use of single DNA molecules for serial single base extension, which are detected using FRET to improve signal-to-noise ratio.

(iii) *DNA sequencing using RNA polymerase*. Recently, a sequencing method was proposed that involved resolution of the motion of a processive RNA polymerase enzyme. This method involves tracking of transcription by single molecules of RNA polymerase (RNAP). A pair of optical traps levitates two polystyrene beads, one attached to RNAP and other to the distal end of the template being sequenced. Motion of RNAP during transcription changes the length of the DNA segment joining the two beads, which can be recorded with precision, providing single base-pair resolution. When transcription is carried out with one of the four nucleotides (NTPs) at a very low concentration, RNAP will pause if it needs the limiting nucleotide. In this manner if four transcription reactions are set up, each with a different rate limiting NTP, RNAP will pause in three of the four reactions, and this will be recorded as time. The records of four reactions can then be aligned to obtain the DNA sequence. The success of this method has recently been demonstrated¹⁸.

Sequencing by ligation (SBL)

A new generation DNA sequencing system, employing a novel approach (sequencing by ligation or SBL), earlier developed by Agencourt Personal Genomics (APG), is also being launched by Applied Biosystems, who has acquired APG. The approach is based on what has been described as Supported Oligo Ligation Detection (SOLiD™) process and may compete with the following two new generation DNA sequencing systems already launched and earlier described in this article: (i) the Roche Diagnostics/454 Life Sciences Genome Sequencer FLX system, and (ii) the Illumina/Solexa Genome Analyser.

SOLiD™ from Applied Biosystems

The above SOLiD™ of Applied Biosystems involves the following five steps: (i) *sample preparation*, which

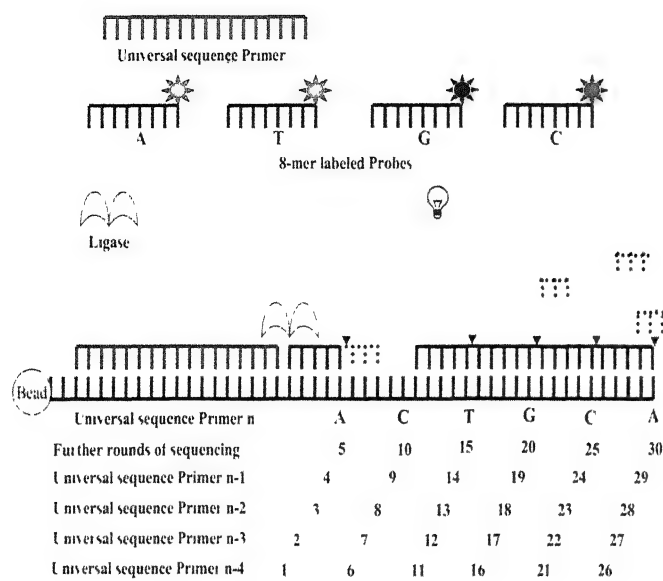


Fig 6 - A representation of the strategy used in Supported Oligo Ligation Detection (SOLiD™) process that is used in DNA sequencing system launched by Applied Biosystems. On the top are shown (i) a 17-mer universal primer that pairs with adapter attached to each fragment to be sequenced (these adapter ligated fragments are each attached to a microbead and em-PCR amplified), (ii) four 8-mer probes differing from each other only at the 5th interrogating base. The universal primer is ligated with each of the four probes separately, and the four primer-probe fragments are annealed serially in order with each adapter-ligated and -amplified fragment. After each round of annealing, the probe is cleaved at the 5th base, to allow annealing of another primer probe fragment. The fluorescent dyes allow identification (base-calling) of bases on positions 5th, 10th, 15th, 20th in order. In subsequent rounds the primers of length n-1, n-2, n-3, n-4, are used, so that bases at positions 4, 9, 14, 19 in the second round, bases at positions 3, 8, 13, 18 in the third round, bases at positions 2, 7, 12, 17 in the fourth round and bases at positions 1, 6, 11, 16 in the fifth round are identified. This will thus give the complete sequence of the fragment in five rounds of annealing and base-calling.

involves development of a genomic library with DNA fragments (~2kb), each with adapters of known sequence ligated at its ends to facilitate designing primers for PCR reactions; (ii) *em-PCR and substrate preparation*, which involves amplification of solitary DNA fragments on individual microbeads using emulsified DNA; (iii) *ligation reaction*, which involves ligation of universal sequencing primer with each of the four 8-base long oligo probes (the fifth base being an interrogating base), each labeled with a fluorescent dye; the ligated primer-probe oligos bind with the adapter-ligated template DNA (probes are used in a set of four

oligos, which differ only at the fifth position; the four oligos are distinguished by four different fluorescent dyes used for labeling); (iv) *imaging and base-calling*, which involves imaging facilitated by the fluorescent dye label that identifies the fifth interrogating base (an activity described as base-calling); the cycle involving ligation, imaging/base-calling and cleavage continues and allows identification of bases at positions 5, 10, 15, 20, 25.....5n (Figure 6). The intervening bases (1-4; 6-9; 11-14; 16-19; 21-24, etc.) are identified through sequential rounds of sequencing using four additional primers with lengths n-1, n-2, n-3 and n-4. The process allows simultaneous reading of millions of DNA fragments in a massively parallel manner.

Nanopore sequencing

Back in 1989, UCSC Professor David Deamer first conceived of making nanopores through which a single strand of DNA would pass at a time and as the strand passed through the nanopore, its changing electrical pattern would be used to read each successive DNA base via sensors built into the nanopore structure (Figure 7). This approach holds the potential of allowing for miniaturization, elimination of lots of expensive reagents, and to speed sequencing by many orders of magnitude. The detection schemes being developed will transduce the different chemical and physical properties of each base into a characteristic electronic signal. Nanopore sequencing has the potential of reading very long stretches of DNA at rates exceeding 1 base per millisecond

The physicists used mathematical calculations and computer modeling of the motions and electrical fluctuations of DNA molecules to determine how to distinguish each of the four different bases (A, G, C, T) that constitute a strand of DNA. They based their calculations on a pore about a nanometer in diameter made from silicon nitride-a material that is easy to work with and commonly used in nanostructures-surrounded by two pairs of tiny gold electrodes. The electrodes would record the electrical current perpendicular to the DNA strand as the DNA passed through the pore. Because each DNA base is structurally and chemically different, each base creates its own distinct electronic signature. The nanopore DNA sequencing method has the potential of having fewer errors than other methods. It should be possible to sequence strands of DNA that are tens of thousands of base pairs in length (possibly as long as an entire gene), in one pass through the nanopore.

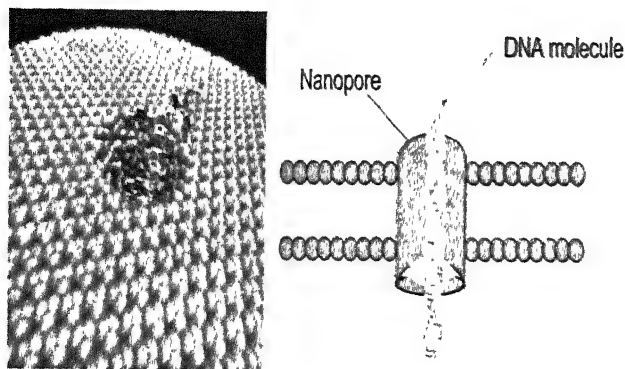


Fig. 7 - The device and the approach proposed in nanopore sequencing that is likely to be used in future. (a) surface view of a nanopore sequencing device; (b) a single DNA molecule travels through the pore; the chemical and physical properties of each base are converted into a characteristic electronic signal to be read by the electrodes fitted in the nanopore (<http://ucsdnews.ucsd.edu/newsrel/science/sfastdna.asp>).

Previous attempts to sequence DNA using nanopores were not successful because the twisting and turning of the DNA strand introduced too much noise into the signal being recorded. The new idea takes advantage of the electric field that drives the current perpendicular to the DNA strand to reduce the structural fluctuations of DNA, while it moves through the pore, thus minimizing the noise. The researchers, however, caution that there are still hurdles to overcome because no one has yet made a nanopore with the required configuration of electrodes, but they think it is only a matter of time before someone successfully assembles the device. The nanopore and the electrodes have been made separately, and although it is technically challenging to bring them together, the field is advancing so rapidly that they think it should be possible in the near future.

ChIPSeq for genome-wide maps of protein binding and histone modification sites (one genome with several epigenomes)

The high-throughput low-cost DNA sequencing platforms discussed above are also being used for a variety of other studies. One such application is the study of genome-wide interactions between a specific protein and genomic DNA, or else the study of the epigenome in different cell/tissue types, each type exhibiting a specific histone modification at specific sites of genomic DNA. In either case, ChIPSeq technique

has been used, in which an antibody raised against the protein or the modified histone of interest is used to selectively coimmunoprecipitate the DNA associated with the protein/histone of interest. The DNA is recovered from DNA-protein complexes and is used for high-throughput DNA sequencing to allow identification of DNA sites associated with specific protein or the modified histone¹⁹. The technique has been successfully used for identification of 1946 locations in human genome for binding of transcription factor NRSF (neuron-restrictive silencer factor)²⁰. Similarly, in another study, 41,582 and 11,004 putative STAT1-binding regions were identified in stimulated and unstimulated human cells respectively²¹. In both these studies, Solexa/Illumina 1G machine was used for high-throughput sequencing of millions of small DNA fragments of ~30 bases length that were ideal for characterizing ChIP derived fragments.

In July/August, 2007, two additional reports were published, where Solexa 1G machine was used for construction of whole-genome maps of chromatin states for a variety of mouse and human cells involving different histone modifications (methylation of different lysine and arginine residues of H3 histone). In one of these studies, sequences of four billion bases of immunoprecipitated DNA were used to generate genome-wide chromatin-state maps of mouse embryonic stem cells, neural progenitor cells and embryonic fibroblasts²². H3K27me3 was found to be an important mark that discriminated between genes that are expressed or poised to express and those which are repressed. Using single nucleotide polymorphisms (SNPs), the chromatin states could also be read in an allele-specific manner. Similarly, in the other study of human epigenomes, high-resolution maps of the distribution of 20 histone lysine and arginine methylations as well as histone variant H2A.Z were generated²³. Typical patterns of histone methylations were identified at promoters, insulators, enhancers, and transcribed regions; monomethylations of H3K27, H3K9, H4K20, H3K79, and H2BK5 were associated with gene activation, while trimethylations of H3K27, H3K9, and H3K79 were linked to repression.

A comparison of three new generation sequencing systems

A comparison of three sequencing systems that became available in 2007 is presented in Table 1, which shows superiority of Solexa and ABI SOLiD over 454

Genome Sequencer FLX in terms of time, cost, precision, and ease of using. However, the only disadvantage with these systems is the read length, which is only 25-36 bases, which makes it relatively unsuitable for de novo whole genome sequencing, and renders them suitable for only resequencing, transcript discovery and for whole genome ChIPSeq. Therefore, questions have been raised whether; de novo accurate assembly can be achieved with sequences as small as only 25-26 bp long at an acceptable computational cost.

We know that for the assembly of sequence fragments of ~500 bp in length, a variety of sophisticated assembly algorithms have been available in the past and are still being used, but short-read assembly algorithms for de novo whole genome sequencing were not available. As the demand for such algorithms increased with the availability of new generation of Solexa and ABI SOLiD sequencing systems, two recent papers reported availability of algorithms SSAKE²⁴ and SHARCGS²⁵, which are claimed to facilitate assembly of sequences that are 25-36 bp long. Since SSAKE was found to allow some misassemblies, SHARCGS was designed to overcome this problem. With the availability of these algorithms, the main hurdle of using Solexa and ABI SOLiD for de novo whole genome sequencing has largely been overcome. With the availability of these softwares and others to be developed, the new generation sequencing systems will certainly replace the Sanger sequencing technology for even the de novo whole genome sequencing (WGS).

Table 1 - A comparison of three new generation DNA sequencing platforms

Features	454 FLX	Solexa	ABI SOLiD
Read length	250bp(350)	25-36bp (50)	25bp (30)
Number of reads/run	420K	~40M	40M
Data output	~100Mb	~1Gb	~3Gb
Cost per run	~\$8,000	~\$3,000	~\$6,000
Cost per Gb	~\$80,000	~\$3,000	~\$3,000
Time per run	8 hours	3 days	3 days
Run time to 1Gb	10 days	2-3 days	7- 10 days
Ease of use	Most difficult	Least difficult	Difficult
Error	0.5%	0.2%	0.1%

Conclusions

The development of low-cost ultrafast DNA sequencing methods has witnessed a revolution in the recent past. At least three new generation sequencing systems have already been launched, one each by 454 Life Sciences/Roche (based on pyrosequencing), Solexa/Illumina (based on cyclic reverse termination) and ABI or Applied Biosystems (based on sequencing by ligation or SOLiDTM). Solexa Genome Analyzer can generate 1 Gb and ABI SOLiD can generate 2-4 Gb sequence in one single run. The 454/Roche system, which is said to be half as fast has also been utilized to sequence the genome of James Watson in the early months of 2007. Several other companies are developing even faster technologies that are not being publicly discussed. Among these, several single-molecule sequencing methods that are being examined for their use in DNA sequencing rely on measurement of either the motion of a DNA/RNA polymerase, or the current across single DNA molecule traversing through a nanopore, or the fluorescence emitted from an individual nucleotide. These, methods, however, are in early stages of development, and several problems associated with them need to be overcome before these can be utilized for actual DNA sequencing for genomics research. If not in the near future, some of these single-molecule sequencing methods should also become available in the long-term.

The above developments have already brought down (or are likely to bring down by 2010) the cost of whole human genome sequencing to \$ 100,000. but bringing it down to the targeted \$ 1000 still remains a challenge. If the sequencing of human genome is brought down to the targeted \$ 1000, this will translate to sequencing a maize genome at an equivalent cost of \$ 1000 and sequencing a rice genome at a cost of \$ 135, thus making it feasible to utilize this technology on a large-scale for applied plant genomics also. In any case, it is certain that the state-of-the-art of DNA sequencing technology will dramatically change within the next 2-5 years, thus bringing about a revolution in applied genomics research.

Acknowledgements

Thanks are due to Ch Charan Singh University, Meerut, for providing the facilities and to the Indian National Science Academy (INSA) for the award of a position of INSA Honorary Scientist, during the tenure of which this article was written.

References

- Collins FS, Morgan M, Patrinos A. The human genome project: lessons from large-scale biology. *Science* 2003; **300**: 286-290.
- Blow N. The personal side of genomics. *Nature* 2001; **449**: 627-632.
- Shendure J, Mitra RD, Varma C, Church GM. Advanced sequencing technologies: methods and goals. *Nat Rev Genet* 2004; **5** : 335-344.
- Metzke ML. Emerging technologies in DNA sequencing. *Genome Research* 2005; **15** : 1767-1776.
- Shaffer C. Next-generation sequencing outpaces expectations. *Nature Biotechnol* 2007; **25** : p. 149.
- Drmanac S. Accurate sequencing by hybridization for DNA diagnostics and individual genomics. *Nature Biotechnol* 1998; **16** : 54-58.
- Drmanac R *et al.* DNA sequencing by hybridization with arrays of samples or probes. *Methods Mol Biol* 2001; **170**: 173-179.
- Gupta PK, Joy JK, Prasad M. DNA chips, microarrays and genomics. *Curr Sci* 1999; **77** : 875-884.
- Patil N, Berno AJ, Hinds DA, Barrett WA, Doshi JM, Hacker CR, Kautzer CR, Lee DH, Marjoribanks C, McDonough DP, Nguyen BTN, Norris MC, Sheehan JB, Shen N, Stern D, Stokowski RP, Thomas DJ, Trulson MO, Vyas KR, Frazer KA, Fodor SPA, Cox DR. Blocks of limited haplotype diversity revealed by high-resolution scanning of human chromosome 21. *Science* 2001; **294** : 1719-1723.
- Aborn JH, El-Difrawy SA, Novotny M, Gismondi EA, Lam R, Matsudaira P, McKenna BK, O'Neil T, Slrcchon P, Lhrlich DJ. A 768-lane microfabricated system for high-throughput DNA sequencing. *Lab Chip* 2005; **5** : 669-674.
- Ju J, Ruan C, Fuller C, Glazer A, Mathies R. Fluorescence energy transfer dye-labeled primers for DNA sequencing and analysis. *Proc Natl Acad Sci USA* 1995; **92** : 4341-4351.
- Lee LG, Spurgeon SL, Heiner CR, Benson SC, Rosenblum BB, Menchen SM, Graham RJ, Constantinescu A, Upadhy KG, Cassel JM. New energy transfer dyes for DNA sequencing. *Nucleic Acids Res* 1997; **25** : 2816-2822.
- Lewis EK, Haaland WC, Nguyen F, Heller DA, Allen MJ, MacGregor RR, Berger CS, Willingham B, Burns LA, Scott GBI, Kittrell C, Johnson BR, Curl RF, Metzker ML. Color-blind fluorescence detection for four-color DNA sequencing. *Proc Natl Acad Sci USA* 2005; **102** : 5346-5351.
- Margulies M, Egholm M, Altman WE, Attiya S, Joel S, Bader JS, Bemben LA, Berka J, Braverman MS, Chen Y-J, Chen Z, Deweli SB, Du L, Fierro JM, Gomes XV, Godwin BC, He W, Helgesen S, Ho CH, Irzyk GP, Jando SC, Alenquer MLI, Jarvie TP, Jirage KB, Kim J-B, Knight JR, Lanza JR, Leamon JH, Lefkowitz SM, Lei M, Li J, Lohman KL, Lu H, Makhijani VB, McDade KE, McKenna MP, Myers EW, Nickerson E, Nobile JR, Plant R, Puc BP, Ronan MT, Roth GT, Sarkis GJ, Simons JF, Simpson JW, Srinivasan M, Tartaro KR, Tomasz A, Vogt KA, Volkmer GA, Wang SH, Wang Y, Weiner MP, Yu P, Begley RF, Rothberg JM. Genome sequencing in microfabricated high-density picolitre reactors. *Nature* 2005; **437** : 376-380.
- Green RE, Krause J, Ptak SE, Briggs AW, Ronan MT, Simons JF, Du L, Egholm M, Rothberg JM, Paunovic M, Paabo S. Analysis of one million base pairs of Neanderthal DNA. *Nature* 2006; **444** : 330-336.
- Braslavsky I, Hebert B, Kartalov E, Quake SR. Sequence information can be obtained from single DNA molecules. *Proc Natl Acad Sci USA* 2003; **100** : 3960-3964.
- Levene MJ, Korlach J, Turner SW, Foquet M, Craighead HG. Zero-mode waveguides for single-molecule analysis at high concentrations. *Science* 2003; **299** : 682-686.
- Greenleaf WJ, Blocks SM. Single-molecule, motion-based DNA sequencing using RNA polymerase. *Science* 2006; **313** : p. 801.
- Madris ER. ChIP-seq: welcome to the new frontier. *Nature Methods* 2007; **8** : 613-614.
- Johnson DS, Mortazavi A, Mayers RM, Wold B. Genome-wide mapping of in vivo protein-DNA interactions. *Science* 2007; **316** : 1497-1502.
- Robertson G, Hirst M, Bainbridge M, Bilenky M, Zhao Y, Zeng T, Euskirchen G, Bernier B, Varhol R, Delaney A, Thiessen N, Griffith OL, He A, Marra M, Snyder M, Jones S. Genome-wide profiles of STAT1 DNA association using chromatin immunoprecipitation and massively parallel sequencing. *Nature Methods* 2007; **4** : 651-657.
- Mikkelsen TS *et al.* Genome-wide maps of chromatin state in pluripotent and lineage-committed cells. *Nature* 2007; **448** : p. 553.
- Barski A, Cuddapah S, Cui K, Roh TY, Schones DE, Wang Z, Wei G, Chepelev I, Zhao K. High-resolution profiling of histone methylations in the human genome. *Cell* 2007; **129** : 823-837.
- Warren RL, Sutton GG, Jones SJM, Holt RA. Assembling millions of short DNA sequences using SSAKE. *Bioinformatics* 2007; **23** : 500-501.
- Dohm CJ, Lottaz C, Barodina T, Himmelbauer H. SHARCGS, A fast and highly accurate short-read assembly algorithm for de novo genomic sequencing. *Genome Res* 2007; **17** : 1697-1706.

Major diseases and the defence mechanism in giant freshwater prawn, *Macrobrachium rosenbergii* (de Man)

SHAILESH SAURABH and P.K. SAHOO*

Central Institute of Freshwater Aquaculture, Fish Health Management Division, Kausalyaganga, Bhubaneswar-751 002, India.

e-mail : pksahool@hotmail.com

Received September 8, 2006, Accepted May 9, 2007

Abstract

The giant freshwater prawn, *Macrobrachium rosenbergii* de Man or scampi is an economically important farmed crustacean species all over the world. India contributes ~ 20% to the world's scampi aquaculture production, being ranked second in the world. In recent years, the rapid expansion and intensification of culture practices have brought several diseases of infectious and non-infectious aetiologies. The increased globalization of trade and transboundary movement of broodstock and postlarvae, the unanticipated interactions between cultured and wild populations, poor biosecurity measures, lack of awareness on emerging pathogens, climate change, misuse of drugs and antibiotics and other human-mediated movement of aquaculture produce and products are playing key role in occurrence, spread and outbreaks of diseases. The main causative agents of the infectious diseases are viruses, bacteria, rickettsia, fungi and protozoa. In past few years, nodavirus is causing a devastating production loss to scampi industry in many countries including India. So disease control is becoming a priority. The defence mechanism of giant freshwater prawn, *M. rosenbergii* de Man. is poorly understood. The knowledge of the functioning of its defence system is of extreme importance. The stimulation of this system is considered as a potential intervention strategy in scampi culture to overcome the infectious diseases. This review focuses on the recent information of major diseases of giant freshwater prawn, *M. rosenbergii* de Man. and the related defence mechanism which may be of help for sustainable development of fast-growing scampi industry.

Key words : *Macrobrachium rosenbergii*, major diseases, defence mechanism

Introduction

Aquaculture plays a major role in increasing fish and shellfish production in tropical countries like India. The vast aquatic resources and conducive environment of India favour rapid expansion of freshwater

सारांश

मीठाजल महाकायझींगा, मैक्रोब्रैकियम रोजेनबर्गाई या स्कैम्पी संपूर्ण विश्व में आर्थिक रूप से एक महत्वपूर्ण क्रस्टेशियन पालन प्रजाति है। संपूर्ण विश्व के कुल स्कैम्पी उत्पादन में भारत ~ 20% की भागीदारी करते हुये द्वितीय स्थान पर आता है। हाल के वर्षों में लगातार बढ़ते हुये पालन क्षेत्र एवं सघनीकरण के कारण इनमें अनेक तरह के सक्रामक एवं असक्रामक रोग भी प्रकट हो रहे हैं। इसके अतिरिक्त बढ़ता हुआ विश्व व्यापार, प्रजनकों एवं पोस्टलार्वा का देश की सीमाओं से बाहर पालाया, पालतू एवं जंगली प्रजातियों का अप्रत्याशित मिलन, कमजोर जैव सुरक्षा, प्रकट होने वाले रोगाणुओं के बारे में अनभिज्ञता, जलवायु परिवर्तन, औषधियों एवं एन्टीबायोटिक का दुरुपयोग, एक्वाकल्चर, उत्पाद एवं उत्पादन का मानव द्वारा परिगमन, विभिन्न रोगों की उत्पत्ति, इनके फैलने तथा वृहद रूप धारण करने में महत्वपूर्ण भूमिका निभा रहे हैं। सक्रामक रोग प्रमुखतः वाइरस, बैक्टीरिया, रिकेट्सिया, कवक तथा प्रोटोजोआ द्वारा उत्पन्न होते हैं। भारत सहित विश्व के अनेक देशों में नोडोवाइरस के कारण पिछले कुछ वर्षों में स्कैम्पी उत्पादन में अत्यधिक क्षति हुई है। अतः बीमारियों की रोकथाम करना एक प्राथमिकता होती जा रही है। मीठाजल के महाझींगा मैक्रोब्रैकियम रोजेनबर्गाई की प्रतिरोधी व्यवस्था का ज्ञान अत्यधिक सकुचित है, परन्तु यह अत्यधिक महत्वपूर्ण है। इस तथ्य के उद्दीपन मात्र से स्कैम्पी के सक्रामक रोगों से बचाव संभावित है। प्रस्तुत आलेख में, मीठाजल महाझींगा के प्रमुख रोगों से संबंधित नवीनतम सूचनाएँ तथा इनसे संबंधित प्रतिरोधी व्यवस्थाएँ वर्णित हैं, जो त्वरित रूप में बढ़ते स्कैम्पी उद्यम के सतत् विकास में सहायक हो सकती हैं।

सांकेतिक शब्द : मैक्रोब्रैकियम रोजेनबर्गाई, प्रमुख रोग, प्रतिरोधक तंत्र।

prawn farming activities. The giant freshwater prawn, *M. rosenbergii*, popularly known as scampi, is the largest and fastest growing freshwater prawn species. It has received considerable attention in recent years due to higher profit realization per unit area^{1, 2, 3}. The ever-increasing demand of the species in

the international market further provides enormous scope for expansion of its cultivation. About four million ha of impounded freshwater bodies in various states of India offer a great potential for freshwater prawn culture. Its farming has drastically increased within the last five years. The production in India is recorded to the tune of 42,820 tonnes. It is ranked second in the world⁴. Scampi is exported to European Union, Japan, USA and other countries. However, concomitant with the rapid expansion and intensification of scampi farming, the disease problems have been causing serious concern.

In any successful farming system, there should always be a proper balance between host, pathogen and environment. In intensive system or high input system, there is every chance of disease occurrence if there is no proper harmony among the three factors. India has already experienced the plummeting of most profitable shrimp aquaculture industry due to white spot disease. In recent years, due to adoption of unscientific and highly intensive farming practices, the freshwater prawn farming in the country has suffered a major setback due to occurrence of a viral disease, caused by *Macrobrachium rosenbergii* nodavirus (*MrNV*)⁵⁻⁸. It has occurred due to import of seed of *M. rosenbergii* from disease-affected countries. The virus has affected the hatcheries and nursery tanks in southern parts of the country causing huge economic losses⁹. This example demonstrates the possible dramatic and unpredicted consequences of transferring infected seeds. Although there is no report on the transfer of this pathogen to wild stock, it is most likely that the pathogen might have escaped to the wild. Therefore, the knowledge of the major diseases and defence mechanism of the freshwater prawn is the need of the hour to minimize or prevent the outbreak of such diseases and to ensure long term survival of scampi industry. In this contribution, an attempt has been made briefly to outline the major diseases and the defence mechanism in the giant freshwater prawn, *M. rosenbergii* de Man, which may be useful for the aquaculturists. The recent trends in scampi aquaculture are towards increased intensification and commercialization of production. Like other fish or animal species, with the increase in the aquaculture activities of scampi there is a likelihood of intensification of some major diseases, which are becoming the bottleneck for sustainability of this industry.

Major diseases of the giant freshwater prawn, *M. rosenbergii*

The giant freshwater prawn *M. rosenbergii* de Man, is generally considered as a less susceptible species to disease problems as compared to penaeid shrimp. However, due to intensification of aquaculture practices and poor quarantine, various diseases, caused by pathogens and nutritional, physiological and environmental factors are often reported in hatcheries and farms. They seriously affect the commercial production. Some of the major diseases of the giant freshwater prawn are listed as follows:

VIRAL DISEASES

White Tail Disease (WTD)/White Muscle Disease (WMD)

The WTD was first observed in Guade Loupe island in 1995. Later on it was reported from Martinique island (French West Indies), Taiwan, Peoples Republic of China and India⁵⁻¹³. The disease is spreading rapidly causing worldwide huge economic loss to scampi industry. The pathogen was identified to be a virus, named as *M. rosenbergii* nodavirus (*MrNV*). *MrNV* is a small icosahedral non-enveloped virus, 26-27 nm in diameter, present in the cytoplasm of connective tissue cells. It has since been reported that *MrNV* is associated with a small satellite virus, called extra small virus (XSV)^{11, 12, 14, 15}. The disease affects hatchery-reared PL as well as nursery-reared early juveniles. The affected individuals show signs of whitish tail or milky-white muscle, leading to 100% mortality. The virus is transmitted vertically from brooders to larvae and PL⁶. Sudhakaran *et al.*¹⁶ reported that marine shrimp act as reservoir host of *MrNV* and XSV.

The clinical signs primarily indicate the disease but confirmatory diagnosis is done by transmission electron microscopy (TEM)¹⁴. Other diagnostic methods like double sandwich enzyme-linked immunosorbent assay (DS-ELISA)¹⁴, triple antibody enzyme-linked immunosorbent assay (TAS-ELISA)¹⁷, dot-blot hybridization, *in situ* hybridization, reverse transcriptase-polymerase chain reaction (RT-PCR)^{15, 18, 19}, multiplex RT-PCR (MRT-PCR)^{20, 21}, nested PCR²² and loop mediated isothermal amplification (LAMP)²³ can also be used for detection of *MrNV* and XSV in scampi. Among this nested RT-PCR has been proved to be highly sensitive diagnostic technique, which has the detection

limits of 0.01 fg of total viral RNA corresponding to four viral particles²².

Infectious Hypodermal and Haematopoietic Necrosis Virus (IHHNV)

Infectious hypodermal and haematopoietic necrosis virus is the smallest (approximately 22 nm diameter) of the known penaeid shrimp viruses^{24, 25}. It is a non-enveloped, icosahedral virus²⁶. The virus was first detected in the juveniles of *Litopenaeus vannamei* Boone. It causes acute epizootics with up to 90% mortality^{27, 28}. IHHNV also causes a chronic disease called runt deformity syndrome in *L. vannamei* Boone and *Penaeus monodon* Fabr.^{29, 30, 31}. However, it was thought that the virus does not cause lethal infections in *M. rosenbergii* de Man, but recent reports of IHHNV infections in *M. rosenbergii* de Man. have shocked the scampi industry in Taiwan³².

This emerging disease particularly encountered in postlarvae and juveniles of *M. rosenbergii* de Man. causes 80%-100% mortality. Affected prawns show growth retardation and deformities of the fourth to sixth abdominal segments and telson, muscular atrophy, associated with reddish discolouration of the cuticle. Histologically, the striated muscles of the affected abdominal segments show Zenker's necrosis. Muscular lysis is also seen in the affected fibres. Nevertheless, no inclusion bodies are detected in any organ. The confirmatory diagnosis of this disease is performed by PCR assays and *in situ* hybridization³².

White Spot Syndrome Virus (WSSV)

White spot syndrome virus (WSSV)/white spot syndrome baculovirus (WSBV) is the causative agent of a disease that decimated cultured penaeid shrimp populations and inflicted severe economic damage throughout the world including India^{25, 33-37}. Though *M. rosenbergii* de Man. was thought to be unaffected by this virus, but signs closely resembling WSSV have been observed on its exoskeleton³⁸. Peng *et al.*³⁸ established WSBV as the causative agent of WSS in the giant freshwater prawn by using polymerase chain reaction (PCR) with WSBV-specific primers. Larvae, PL, juveniles and adults were found to be PCR-positive. The amplified PCR product was found to be similar to that of natural infected penaeid WSSV, hence great care should be taken to reduce or prevent the epizootics of WSSV in freshwater scampi culture.

In experimental studies by Rajendran *et al.*³⁷, a WSS virus obtained from *P. monodon* Fabr. from the south-east coast of India was used to infect *M. rosenbergii* but showed 100% survival and no clinical symptoms up to 70 days. In a similar study, Sahul Hameed *et al.*³⁹ used a viral inoculum obtained from *P. monodon* Fabr. with WSSV to test the relative susceptibility of three freshwater prawn species. All species tested had significant mortality except *M. rosenbergii* de Man. even when the latter was given multiple injections of increasing dosage. In contrast, Kiran *et al.*⁴⁰ reported that the different life-stages of *M. rosenbergii* de Man. are susceptible to WSSV.

Macrobrachium Muscle Virus (MMV)

Tung *et al.*⁴¹ reported *Macrobrachium* muscle virus infection in *M. rosenbergii*, cultured in Taiwan. The virus is temporarily named MMV until its taxonomic position is ascertained by analyzing the genomic structure. This virus mostly affects postlarvae (PL) with epizootics similar to idiopathic muscle necrosis (IMN) syndrome with exceptional addition of dense cytoplasmic inclusion bodies in the affected muscles. Electron microscopy shows icosahedral virus particles of 22.9±3.6 nm size. MMV infects giant freshwater prawn in July and December within 10 days of moving PL to outdoor ponds. Moribund prawns grossly exhibited non-transparent muscle in abdominal segments 2-6. The affected prawns commonly displayed progressively weakened swimming ability and an inclination to stay on vegetation or the pond bank. A mortality of 50%-75% is seen within 2 weeks after transporting the postlarvae to grow-out ponds⁴¹.

Macrobrachium Hepatopancreatic Parvo-like Virus (MHPV)

Macrobrachium hepatopancreatic parvo-like viruses have not received much attention among entrepreneurs and researchers because they are not causing any serious epizootics in *M. rosenbergii* culture. The definitive diagnosis of MHPV requires the histological demonstration of a characteristic inclusion body within the hypertrophied nucleus of the affected cells of the hepatopancreatic epithelia⁴².

Macrobrachium HPV is similar to the hepatopancreatic parvo-like virus described from penaeid shrimp in terms of the target cells attacked, inclusion body characteristic and nucleic acid type. However,

it differs from shrimp HPV by an *in situ* hybridization test for genomic HPV that shows no positive reaction with MHPV preparations⁴³.

BACTERIAL DISEASES

Several diseases of bacterial origin are frequently reported in hatcheries and grow-out ponds. However, the type and levels of bacterial populations associated with farmed *M. rosenbergii* de Man. are useful indicators of quality and safety of prawns⁴⁴. It is reported that larval stages are most susceptible to a bacterial disease as a result of high stocking density, long rearing period and organic load accumulation at the pond bottom. Some of the bacterial diseases frequently encountered in scampi farming are as under.

Vibriosis

Vibriosis has been identified as a major challenge to scampi industry worldwide^{43, 45-48}. Jayaprakash *et al.*⁴⁸ reported 9 species of *Vibrio* associated with *M. rosenbergii* larvae. The major *Vibrio* sp. infecting scampi includes *V. cholerae*, *V. nereis*, *V. alginolyticus*, *V. vulnificus*, *V. mediterranei*, *V. parahaemolyticus*, *V. splendidus*, *V. fluvialis*, *V. mimicus*, *V. harveyi* etc. Among these, *V. cholerae*, *V. alginolyticus* and *V. vulnificus* are predominant^{48, 49}. In general, *Vibrio* sp. are prevalent on eggs, larvae and post larvae of freshwater prawns⁵⁰ and their increasing number during culture period is a serious concern^{51, 52}.

It has been reported that luminescent bacterium *V. harveyi* causes massive losses of scampi larvae in hatcheries^{53, 54}. The unique clinical sign of this disease is the luminescence of affected larvae that can be observed at night. Infected larvae also show fouling, opacity, slow swimming, aggregation and mortality. Mortality may reach 100% and may cause complete failure of the crop, resulting in severe economic loss to farmers.

The use of antibiotics to control vibriosis in shrimp/prawn hatcheries has been documented⁵⁵. However, prophylactic use of antibiotics can lead to the emergence of antibiotic resistance in bacteria⁵⁶. As an alternative management measure, the introduction of selected bacterial cultures/products as probiotics with antagonistic properties has been proposed and applied⁵⁷. Misra *et al.*⁵⁸ reported that bath immunostimulation with β -glucan provides better protection against *Vibrio* infection in *M. rosenbergii* de Man. Jayaprakash *et al.*⁴⁷ reported that

marine bacterium, *Micrococcus* MCCB 104 show antagonistic properties against most of the *Vibrio* sp. associated with *M. rosenbergii* de Man. larval rearing system. Anas *et al.*⁵² reported that chitosan can be used as a prophylactic biopolymer for protecting prawn larvae from vibriosis. Chitosan in the larval rearing system may function as a *Vibrio* growth depressant. As the risk of infection is directly related to pathogen density, depressed cell counts may help to prevent larval vibriosis. Further, chitosan is recognized as an immunostimulant in fish⁵⁹; hence, it may also be worthwhile to test it as immunostimulant in freshwater prawn rearing system.

Shell Disease

Shell disease is the most common bacterial disease affecting crustaceans⁶⁰. The disease has been variously referred to as brown spot disease, black spot disease and rust disease. Chitinolytic bacteria such as *Vibrio*, *Aeromonas*, *Pseudomonas*, *Bacillus* and *Benkeia*, which produces extracellular enzymes, often have been associated with this disease^{53, 61, 62}. The disease affects all life stages of *M. rosenbergii* de Man. The presence of one to several focal melanised lesions on the body surface is the most distinct gross sign⁶³. Blackening is usually associated with melanin production that is bacteriostatic in action. The disease is usually seen focally on the gills, carapace, appendages, uropods, telson or body cuticle. The clinical signs are self-limiting and shed with the exuvia in healthy prawns. In severe cases, infection may spread to the epithelium, muscle and viscera resulting in septicemia and mortality⁶⁴.

Often, development of black spot is associated with poor husbandry, overcrowded conditions and poor sanitation⁶⁵. Maintenance of optimum water quality, adequate feeding, less stocking density and hygiene are some important control measures. Antibiotic treatment has also been reported for control of black spot in *Macrobrachium* species⁶⁶. Chemical treatment is not recommended for control of black spot disease in *M. rosenbergii* de Man⁶⁷. Delves-Broughton and Poupard⁶⁸ however, viewed that shell disease does not lend itself to prophylactic and chemotherapeutics control. The best way to control shell disease in prawns thus seems to be thorough environmental management, which reduces stress factors and its regulation^{69, 70}.

Bacterial Necrosis

Bacterial necrosis, first described by Aquacop⁶⁹, is similar to black spot in terms of gross signs but

is restricted to larval phase only. Larval stages IV and V are generally more susceptible showing the necrosis and melanisation of whole body appendages. Other clinical signs of this disease are empty gut, bluish colour of affected larvae and brown spots on antennae and newly formed appendages. Such larvae gradually fall to the bottom of the tank. Mixed bacterial infections have been observed in such cases. Filamentous *Leucothrix* sp. and nonfilamentous bacilli and cocci usually infest on the setae, gills and appendages of the affected freshwater prawns⁷¹.

Bacterial necrosis in *Macrobrachium* larvae may represent a secondary bacterial invasion of the cuticle following some as-yet-undetermined primary insults, which cause disruption of the epicuticle allowing bacterial entry⁶⁷. A diagnosis of bacterial necrosis in larvae of freshwater prawn is made by microscopic demonstration of the characteristic focal to multifocal melanized cuticular lesions of larval appendages⁷².

Development of bacterial necrosis is rapid and if untreated, can be responsible for mass mortality of larvae within a short span of time. Onset of necrosis has been linked to a decrease in larval resistance⁷³. Antibiotic treatment has been reported to be effective for control of bacterial necrosis in *Macrobrachium* larvae⁷¹.

Gram-positive Bacterial Infection

Very few reports are available on infection of Gram-positive bacteria in cultured freshwater prawn *M. rosenbergii* de Man. Brock *et al.*⁷⁴ diagnosed Gram-positive bacteria *Mycobacterium* in a female broodstock kept in captivity for more than 7 years. Cheng and Chen⁷⁵ isolated *Enterococcus*-like bacterium from the musculature of *M. rosenbergii* de Man in Taiwan, which has been identified as *Lactococcus garvieae* by polymerase chain reaction assay and 16s rDNA sequencing⁷⁶. Such infected prawns show necrosis of tissue in the hepatopancreas and musculature. Experimentally it has been proved that high pH and temperature exacerbate infection while low salinity appeared to have a favourable effect upon survival⁷⁷.

RICKETTSIAL DISEASE

Cohen and Issar⁷⁸ reported rickettsial infection of the hepatopancreas of *M. rosenbergii* de Man larvae at stage IV to V resulting in 40% to 95% mortality. Microscopically, the disease was characterized by

atrophy of the hepatopancreas⁷⁹. The infection was successfully transmitted to healthy larvae by exposure to a homogenate of infected larvae⁷⁸. Application of calcium oxide (CaO) at 10 to 15 ppm reduces losses, as did treatment with 10 ppm of either terramycin or furazolidone^{79, 80}.

Considering the frequency of its infection in crustaceans, it is apparent that rickettsial diseases are potentially more important than was once understood⁶³.

FUNGAL DISEASES

Fungal infection of the Phycomycete *Lagenidium* in *M. rosenbergii* de Man hatcheries causes decimation of larval populations in less than 24 h⁸¹. The infection can be readily diagnosed by the observation of an extensive mycelial network visible through the exoskeleton of infected larvae. It is reported that Treflan at 10-100 ppb is effective in controlling of *Lagenidium* infestation in penaeid hatcheries⁸².

Recent work with a large sampling of isolates in Taiwan has helped to remove confusion about the identity of prawn-infecting yeasts^{83, 84}. Yeasts reported to be pathogenic for *M. rosenbergii* de Man, include *Candida sake* I and II, *Pichia anomala*, *Endomyces fibuliger* and *Candida famata*⁸⁵. Su *et al.*⁸⁴ reported that yeast *Metschnikowia bicuspidata* is the major cause of white muscle disease, but the molecular mechanism of its pathogenesis is not known. The imperfect fungus, *Fusarium soleni*, *Saprolegnia*, *Sirolopidium* and other fungal genera may also be associated with prawn mortality^{82, 86}. The yellowish, bluish or greyish colour of the musculature and a reduced resistance to the effects of stressful conditions, are indicative of such diseased animals⁸⁷. The occurrence of the disease has been associated with the accumulation of organic matter in the pond bottom and eutrophication in grow-out ponds. Therefore, possible preventive measures are careful pond preparation, proper feeding, water exchange and aeration etc, which provide congenial environment for freshwater prawn culture.

PARASITIC DISEASES

Microbial Fouling

Microbial epibionts are commonly observed disease agents in *M. rosenbergii*. Epibiont fouling organisms include filamentous bacteria, algae or protozoa common

to the aquatic environment. The presence of epibionts, nonetheless, can be deleterious to freshwater prawn. Fouling by various microorganisms can provoke natatory and feeding impairment during larval and postlarval phases. Heavy infestation of filamentous algae, protozoans and miscellaneous microbes over the body surface of reared animals in clear ponds, diminish the market value of the prawns. Heavy fouling by the chlorophyta *Oedogonium* and the cyanophyta *Lyngbya* can cause lethargic behaviour in *M. rosenbergii* de Man⁸⁸.

The most common protozoans found in freshwater culture are the peritrich ciliates *Zoothamnium*, *Epistylis*, *Vorticella*, *Opercularia*, *Vaginicola*, *Cothurnia* and *Lagenophrys* and the suctorians *Acineta*, *Podophrya*, *Tokophrya* and *Ephelota*^{89, 90}. Larvae of scampi are more susceptible to protozoan infections than adults. These organisms are non-invasive and their presence on the cuticle surface does not trigger the activation of the proPO system, unless a localised mechanical trauma is present. Generally, *Zoothamnium* sp., *Epistylis* sp., *Vorticella* sp. and *Acineta* sp. cause slight opacity in body colour of freshwater prawn. Shedding of the exoskeleton during ecdysis temporarily frees the prawn from these fouling microorganisms. Large animals and blue claw males, which moult less often, are frequently seen with excessive burdens of epibionts. Improvement of water quality is necessary when the outbreaks occur. Treatment with 20-30 ppm formalin is effective and safe in controlling *Zoothamnium* infection of the larvae⁹⁰. Repeated treatment of 2 ppm acetic acid as a 1 min dip is recommended for *Epistylis* sp.⁹¹.

Metazoan Parasites

The infection of metazoan parasites in cultured freshwater prawn is rare. Most reports concern over the occurrence of parasitic isopods and trematode worms in natural populations of other *Macrobrachium* species. Isopods that infest freshwater prawns belong to the family Bopyridae⁹². The asymmetrically shaped female bopyrid is found attached to the branchiostegite wall within the gill chamber and feeds on the host haemolymph by perforating the integument with the help of its mandibles. The symmetric free living dwarf males are found associated with the parasitic females. The presence of the parasite in the gill chamber usually induces a visible swelling and discolouration on the branchiostegite⁹³. Johnson⁹⁴ reported that *Macrobrachium* serves as second intermediate host for the metacercaria of *Carnaeophallus choanophallus*.

Nash⁹⁵ reported the only known trematode infection in *M. rosenbergii* de Man. musculature and described the formation of a haemocytic capsule around the parasite as the result of defence response. Control methods are limited to physical removal of parasites from prawns and prevention of extension of the range of these parasites into new geographic area.

OTHER DISEASES OF UNKNOWN AETIOLOGY

Appendage Deformity Syndrome (ADS)

Recently, a new disease with unusual clinical signs has been reported in the Nellore district, motherland of scampi culture in India. This disease is named appendage deformity syndrome (ADS) because of the physical appearance of affected prawns^{7, 96}. ADS has affected more than 80% of the area under culture in Nellore district.

ADS is a chronic disease associated with late mortality particularly at 4 to 6 months of culture when prawns have reached maturity. The clinical signs of ADS are more prevalent in females than males. The most striking features of the disease are appendage deformity, broken, bent or deformed rostrum, cut antennae, beaded or corrugated appearance of antennules which are more prone to breakage, corrugated appearance of the carapace, poor growth and varied mortality^{7, 97}. Kumar *et al.*⁹⁶ reported that carotenoid supplementation (1g/kg) in the diet significantly reduces the ADS in prawns, both in laboratory and field. The same study also ruled out possibility of involvement of any bacterial or viral agents responsible for causing ADS. Sahoo *et al.*⁷ reported that alterations in the physico-chemical parameters of water might be playing roles as predisposing factors or causal factors for ADS. However, further study is required to confirm the involvement of any pathogen or carotenoid deficiency or water quality as the possible cause of ADS.

Branchiostegal Blister Disease (Balloon Disease)

Pillai *et al.*⁹⁸ reported a branchiostegal blister disease or balloon disease of farmed *M. rosenbergii* de Man. restricted to Nellore region of India. The disease is clinically characterised by the swelling of the gill flap or branchiostegite region and is hence named as 'Branchiostegal blister disease' (BBD) 'or' 'balloon disease' by the farmers⁹⁹. It usually appears in nurseries within

30 days of stocking or in grow-out ponds after transfer from nurseries. The mortality ranges from 70% to 80%. The cause of the disease is unknown but it is thought that suboptimal water quality conditions may be responsible for it.

Idiopathic Muscle Necrosis

Idiopathic muscle necrosis (IMN) is a pathological condition reported from cultured *Macrobrachium* larvae, PL, juveniles and subadult^{64, 100, 101}. This disease is also known as white muscle disease, spontaneous muscle necrosis, muscle opacity or milky prawn disease. It causes massive larval mortalities in hatcheries^{53, 64}. The disease shows focal and multifocal diffused opacity of striated muscles. At the chronic stage of the disease, the necrotic affected area may increase in size and acquire a reddish colour, similar to cooked shrimp, due to decomposition of the muscle tissue. Prawns suffering from chronic IMN do not survive. The mortality rates in culture populations are reported to vary from insignificant to 100%⁶⁷.

Diagnosis of IMN in *Macrobrachium* larvae can be made by gross demonstration of opacity of the abdominal musculature¹⁰². Histopathology is suggested for confirmation of the characteristic focal degenerative and/or regenerative change in the musculature. A specific approach for the control of IMN in *Macrobrachium* zoea is presently not documented. The development of the disease appears to be associated with poor management. IMN has been observed in populations submitted to high stocking rates and stressful environmental conditions such as low dissolved oxygen level, salinity and temperature fluctuations^{1, 53}. The obvious preventive measures include the application of good husbandry to avoid IMN inducing stressors.

Larval Mid Cycle Disease

A syndrome of *Macrobrachium* larvae with the onset in the middle of the larval cycle is mid-cycle larval disease (MCD). It has been reported from Hawaii, Malaysia and Brazil^{42, 103}. Although the implication of many pathogens, toxic compounds or pesticides have been suggested, a precise cause remains undetermined¹⁰³. Mid-cycle disease is probably a disease of multiple aetiology.

The onset of MCD usually occurs by 15 to 22 days when zoea larvae are at stage VI-VII¹⁰³. Clinical

signs include lethargy, a spirally swimming pattern, reduced growth rate, less consumption of *Artemia*, bluish-grey colour and atrophy of the hepatopancreatic epithelium. Larvae lose their appetite. The healthy ones eat moribund individuals showing cannibalism. Destroying the stock, strict attention to hygiene and sanitary conditions are some of the control measures. Brock⁶⁷ reported that reducing the stocking density and improving pond husbandry and sanitation could control the MCD disease.

Exuvia Entrapment Disease

The exuvia entrapment disease (EED) is also known as moult death syndrome (MDS) or metamorphosis moult mortality syndrome. It is characterised by the entrapment of the larvae in the old exoskeleton during ecdysis. This primarily occurs late in the period of larval rearing cycle and most commonly at the metamorphic moult from stage XI to post larvae⁶⁷. Affected larvae are unable to free their appendages, eyes or rostrum from the exuviae during and after moulting and become entrapped. Mortality occurs during unsuccessful attempts by larvae to shed the exuvia. It may reach 80% by the time of metamorphosis⁶⁷. Kanaujia *et al.*¹⁰⁴ reported an exuvia entrapment like disease in Indian river prawn *M. malcolmsonii* Milne Edwards, causing mass mortality in hatchery tanks.

The presumptive diagnosis of EED in *Macrobrachium* larvae is made by high survival up to stage X-XI, many of moribund or dying larvae/PL in late premoult, entrapped in their exuviae, moulted larvae showing severe, lethal appendage deformity which may have apparently stalled in a supernumerary stage XI⁶⁷.

The precise cause of EED is unknown but it is thought that poor water quality, and inadequate nutrition may be responsible for this dreaded disease. Preventive strategies should include use of a new water supply of more suitable quality and dietary enrichments. The moulting process creates severe physiological stress and during this period the disease manifests itself.

Black Gill Disease

Johnson⁸⁹ reported that precipitating chemicals and nitrogenous waste matter present in the water are responsible for melanization of the gills. High amount of ammonia and nitrite in the ponds result in growth suppression and mortality. *Macrobrachium* are more

susceptible to high nitrite and nitrate concentrations than penaeid shrimps¹⁰⁵. Sublethal effects of nitrite could be fatal in chronic exposure and may occur at less than 2 ppm¹⁰⁶. Nitrogenous compound levels in rearing water medium should be routinely monitored. Preventive measures should therefore include feeding adjustment or increased water replacement in rearing tanks or grow-out ponds.

Red Discolouration

Johnson⁹² reported reddish abdominal discolouration affecting adult freshwater prawn in a grow-out pond. The abnormal pigmentation had resulted from the movement of the pigment from chromatophores that had lost their usual integrity. The precise cause of red discolouration is not known but it is thought that excess light, diet and stress may be responsible for this abnormality.

Defence mechanism of the giant freshwater prawn, *M. rosenbergii*

The defence mechanisms of *M. rosenbergii* de Man. and other crustaceans differ from those of the vertebrates. It does not show the production of immunoglobulins. Defence system of giant freshwater prawn may be classified as fixed defences, mobile defences and Pro-phenol oxidase system (PPO) defence. The hard exoskeleton or cuticle of freshwater prawn acts as structural and chemical barrier to several pathogens. It acts as a first line of defence and has the capacity of rapid wound healing which prevents loss of haemolymph. It contains immune substance like PPO¹⁰⁷. Humoral (antisomes) and cellular (haemocytes) factors, which involve the direct participation of circulatory haemocytes and dissolved factors in the plasma, regulate the mobile defence system. Although the defence response in crustaceans lacks the antigen-antibody specificity found in vertebrates, it is nonetheless efficient in recognizing the antigen and taking prompt action to immobilize and eliminate invading microorganisms⁶³.

M. rosenbergii de Man. has an open circulatory system with no equivalents to vertebrate red blood cells, but cells analogous to the white blood cells appear to exist. These cells, called haemocytes, can be grouped into three subpopulations in crustaceans: granular, semigranular and hyaline cells^{108, 109}. The circulating haemocytes of freshwater prawn play an extremely

important role not only by direct sequestration and killing of infectious agents but also by synthesis and exocytosis of a battery of bioactive molecules^{110, 111}. Essentially, the haemocytes execute inflammatory-type reactions such as phagocytosis, haemocyte clumping, production of reactive oxygen metabolites and the release of microbicidal protein^{112, 113}. In crabs, the hyaline cells mainly execute phagocytosis and production of reactive oxygen while in other species, semigranular cells may be phagocytic. *Marsupenaeus japonicus* Bate. is unusual as in this species the granular cells are phagocytic instead of hyaline cells^{114, 115}. There exists difference of opinion on the cell types and their phagocytic function in *M. rosenbergii* de Man. For example, Sung *et al.*¹¹⁶ found all the three cell types (hyaline, semigranular and granular cells), in haemolymph of *M. rosenbergii* de Man. which display atleast some phagocytic activity. Based upon the recent classification of *M. rosenbergii* de Man. haemocytes^{117, 118}, large ovoid haemocytes and undifferentiated round haemocytes might be carrying out the functions of the proPO system, similar to semigranular and granular haemocytes of other crustaceans¹¹⁹.

Phagocytosis can be initiated by receptor-mediated endocytosis or through non-specific hydrophobic interactions of cell membrane with target particles^{120, 121}. It is a common reaction of cellular defence and is generally recognized as a central and important way to eliminate microorganisms or foreign particles. Phagocytosis includes attachment to the foreign body followed by its ingestion and destruction^{122, 123}. In phagocytosis, a form of endocytosis, large particles move into endocytic vesicles called phagosomes. Prawn haemocytes contain cytoplasmic granules and lysozyme¹²⁴. A wide variety of degradative enzymes and antimicrobial peptides are released into the phagolysome following intake of foreign organisms. These enzymes include proteases, nucleases, phosphatases, esterases and lipases¹²⁵.

Several kinds of reactive oxygen intermediates (ROIs) are produced during phagocytosis. Once the invading microorganism infects the host, it activates the host's NADPH-oxidase, which in turn reduces oxygen molecules and subsequently produces several ROIs such as superoxide anion ($O_2^{\cdot -}$), hydrogen peroxide (H_2O_2) and hydroxyl radical (OH) and singlet oxygen (1O_2)¹⁰⁹. These compounds can be toxic to the pathogens¹²⁶. This phenomenon, known as respiratory burst activity plays a vital role in microbicidal

activity¹²⁷. The generation of superoxide anion has been reported in the haemocytes of the shore crab, *Carcinus maenas*¹²⁸, the tiger shrimp, *Penaeus monodon*¹²⁷ and the blue shrimp, *P. stylirostris*⁷⁰, white shrimp *L. vannamei*¹²⁹⁻¹³¹, *Marsupenaeus japonicus*¹¹⁴, *M. malcolmsonii*^{132, 133} and *M. rosenbergii*¹¹⁸. It is also reported that rapid increase of superoxide anions and other ROIs may pose potential cytotoxic problems to the host^{130, 134}. Antioxidant defence mechanisms including NADPH oxidase, superoxide dismutase (SOD), peroxidase and catalase perform the effective and rapid removal of ROIs^{135, 136}.

Superoxide dismutase is an important antioxidant enzyme that scavenges superoxide anion and converts it to molecular oxygen and hydrogen peroxide¹³⁵. They are classified into three distinct groups depending on the metal contents: iron SOD (Fe-SOD), manganese SOD (Mn-SOD) and copper/Zinc SOD (Cu, Zn-SOD). Fe-SOD has been found in prokaryotes and in plants¹³⁷. In decapod crustaceans, cytosolic Mn-SOD (cytMn-SOD) has been identified in tiger shrimp, *P. monodon* Fabr. (GenBank accession no. AAW50395), kuruma shrimp, *M. japonicus* (GenBank accession no. BAB85211), brown shrimp, *Farfantepenaeus aztecus* Ives., grass shrimp, *Palaemonetes pugio* Holthius, blue crab, *Callinectes sapidus* Rathbun.¹³⁸ and *M. rosenbergii* de Man¹³⁶. Mn-SOD has been cloned in lobster, *Palinurus vulgaris*¹³⁹, white shrimp, *L. vannamei* Boone.¹⁴⁰, tiger shrimp *P. monodon* Fabr. (Genebank accession no. B1784454), kuruma shrimp, *M. japonicus* Bate. (Genebank accession no. BAB85211), grass shrimp, *Palaemonetes pugio* Holthuis. (Genebank accession no. AY211084), blue crab, *Callinectes sapidus* Rathbun.^{138, 141} and *M. rosenbergii* de Man.¹⁴² However, in crustaceans, little is known on the cloning and characterization of Cu, Zn-SOD. Recently, Cu, Zn-SOD has been cloned from the hepatopancreas of *M. rosenbergii* de Man.¹⁴³

In addition to phagocytosis, haemocytes also enhance coagulation and encapsulation. They accelerate the production of melanin via the prophenoloxidase system^{144, 145}. Encapsulation, a process wherein layers of cells surround the foreign material, occurs when a parasite is too large to be ingested by phagocytosis. Nodule formation, which is similar to capsule formation, occurs when the number of invading bacteria is high¹⁰⁹. The prophenoloxidase (proPO) activating system is a cascade of enzymes and other proteins, which is contained in the granular and semigranular cells, and can

specifically be activated by several microbial polysaccharides, including β -1,3-glucan from fungal cell walls¹⁴⁶⁻¹⁴⁸ and peptidoglycans¹⁴⁹ or lipopolysaccharides¹⁵⁰ from bacterial cell walls. Additional factors found to activate the proPO system include calcium, sodium dodecyl sulfate (SDS), trypsin, and high temperature^{149, 151-154}. According to Ashida and Soderhall¹⁵¹, these elicitors activate the proPO system through different mechanisms.

The major enzyme produced during proPO system activation is phenoloxidase (PO) that is necessary for the melanization process observed in response to infection and occurs in cuticular wounds or nodules and capsule formation around invading parasites¹⁴⁴. A complex enzymatic cascade that involves activation of proPO to PO induces melanization^{123, 155}. PO is a copper-containing oxidase that catalyses the oxidation of phenolic substances such as L-3, 4- dihydroxyphenylalanine (L-DOPA) to quinones which are then further polymerized non-enzymatically to the black pigment, melanin¹⁵⁶.

Without doubt, the proPO system is a dominant part of the crustacean defence system exerting effects on cell behaviour, liberation and/or activation of functionally important molecules and neutralization of infective agents. Its multiplicity of effects, direct or indirect, not surprisingly, has made it one of the central parts of the crustacean immune system to be targeted for up-regulation by externally administered stimulants¹¹³. The activity of phenoloxidase has been reported from many crustaceans including the brown shrimp, *Penaeus californiensis* Holmes.¹⁵⁷, Sao Paulo shrimp, *Farfantepenaeus paulensis* Perez-Farfante.¹⁵⁸, white shrimp, *Litopenaeus vannamei* Boone.^{129, 131}, tiger shrimp *P. monodon* Fabr.¹⁵⁹, Indian river prawn, *M. malcolmsonii* Milne Edwards.^{132, 133} and giant freshwater prawn, *M. rosenbergii* de Man.^{118, 145}.

Several compounds or associated factors of the proPO system have been found in crustaceans. They include the recognition proteins^{160, 161}, cell adhesive proteins¹⁶²⁻¹⁶⁴, protease inhibitor¹⁶⁵ and proPO activating proteinase^{166, 167}. The biological activity of peroxinectin, a cell adhesive protein, is generated concomitant with the activation of the proPO system¹⁶⁸. During cell adhesion, peroxinectin is synthesized and stored in semi-granular and granular haemocytes in an inactive form. It is released in response to a stimulus and activated outside the cells to mediate haemocyte attachment and spread¹⁶⁸⁻¹⁷⁰. Peroxinectin has multiple functions of degranulation¹¹⁹, encapsular enhancement¹⁷¹, opsonin¹⁷²

and is related to the activity of peroxidase¹⁶². When a foreign particle enters the haemolymph of a host, haemocytes recognize the foreign intruder as non-self and change from non-adhesive cells to adhesive cells to strongly adhere to the foreign target. The semigranular and granular cells attach and spread across the surface of the foreign intruder and subsequently form a multilayered sheath of cells during encapsulation. Therefore, peroxinectin is essential in crustacean cellular defence reaction for enhancement of encapsulation and phagocytosis¹¹⁹.

Peroxinectin has been isolated and purified from crayfish, *Pacifastacus leniusculus* Dana and tiger shrimp, *P. monodon* Fabr. and show cell adhesion and peroxidase activities in the presence of lipopolysaccharide (LPS) or β -1,3-glucans^{163, 168}, suggesting that this protein plays an important role in defence against pathogens¹⁷⁰. Peroxinectin cDNA has been cloned and sequenced from the haemocytes of *P. leniusculus* Dana¹⁶², *P. monodon* Fabr.¹⁶³, *L. vannamei* Boone¹⁷⁰ and *M. rosenbergii* de Man.¹⁷³.

A number of haemolymph factors are associated with defence reaction of *M. rosenbergii* de Man. These factors include naturally occurring or inducible bioactive molecules, which agglutinate and precipitate the non-self particles as well as those that have bactericidal, lytic or bacteriostatic properties. Agglutinins, which cause aggregation or agglutination of foreign particles, have been reported in freshwater prawn^{132, 133}. Agglutinins would help sequestration of invasive organisms from the haemolymph and therefore contribute to disease resistance¹⁷⁴. Lectins, which are generally recognized by their ability to cause haemagglutination are also reported from many crustaceans species including *M. rosenbergii* de Man.¹⁷⁵⁻¹⁸¹ and *M. malcolmsonii* Milne Edwards^{132, 133}. Lectins are multivalent carbohydrate binding proteins that actively participate in cellular and humoral immune response^{182, 183} and are able to agglutinate erythrocytes, bacteria and other cells through interaction with appropriate complementary ligands¹⁸⁴⁻¹⁸⁶. In *M. rosenbergii* de Man. a homodimeric protein of 9.6 kDa per subunit (MrL) has been purified in a single step by affinity chromatography on a column with stroma from rat erythrocytes¹⁸⁷. It is specific for N-acetylated sugar residues, such as N-acetyl-D-neuramic acid (Sialic acid, Neu5Ac), N-acetyl-D-galactosamine (GALNAc) and N-acetyl-D-glucosamine (GLcNA)¹⁸⁷ and shows high affinity for sialylated glycoproteins and glycans¹⁸⁸.

Agundis *et al.*¹⁷⁸ quantified lectin in *M. rosenbergii* de Man. haemolymph by ELISA. Soria *et al.*¹⁸¹ reported that sugars and amino acids present in the haemolymph of prawn also regulate lectin activity and the oxidative burst. It has also been reported that *M. rosenbergii* de Man. lectin (MrL) agglutinates erythrocytes from rat and rabbit, and possess the capacity to agglutinate bacterial species with well known polysaccharide determinants such as *Pasteurella* hemolytic serotype A (Capsular serotype 12), *Bacillus cereus* and *Aeromonas* sp., as well as in a minor proportion, *Escherichia coli* and *Salmonella arizona*¹⁸⁸. MrL has also been identified as a marker for neurodegenerative diseases, such as Alzheimer disease¹⁸⁹.

Clotting factors play a vital role in defence system of crustaceans. Coagulation in crustaceans results from the direct conversion of a soluble fibrinogen (coagulogen) into crosslinked fibrin through the action of a coagulin released by the haemolymph¹¹⁰. The activation of clotting cascade may be triggered by microbial cells such as LPS and β -1,3-glucans, resulting in the formation of a clot that entraps invading microorganisms. This process is also linked to the triggering of proPO activation¹²³.

Strategies for combating diseases in prawn aquaculture

For preventing or controlling infectious diseases in prawn farming, the following possible strategies are to be followed.

- India should implement quarantine rules/protocols stringently and an established international aquatic animal health code on the introduction and transfer of organisms must be followed.
- Asian Regional Technical Guidelines (FAO¹⁹⁰) on health management for the responsible movement of live aquatic animals should be implemented.
- The national strategy on listing pathogens, developing diagnostics, health certification, seed certification, quarantine, hatchery accreditation, institutional-governmental-farmer cooperation may be strengthened.
- Level I diagnostic facilities at the field level (gross examinations), level II diagnostics at state levels (use of parasitological, bacteriological, mycological and histopathological tools) and level III diagnostics at research institute/university/central level (i.e.

laboratory observations using virology, immunology, molecular biology and electron microscopy) should be strengthened.

Biosecurity, disease surveillance, reporting and disease zoning may be planned.

Research should be strengthened with respect to development of easier, sensitive and rapid diagnostics; epidemiological mapping of prawn diseases with special emphasis on interventions, risk factors, forecasting and disease modeling; determining pathways for introduction, establishment and spread of pathogens and understanding detailed pathology of the diseases.

Nevertheless, emphasis should be given on manpower development with respect to education, training, extension and diagnostic services and establishment of regional reference laboratories to cater the need of the prawn farmers.

Conclusions

The intensive culture practices of *M. rosenbergii* de Man occasionally trigger the development of disease outbreaks, which are often explosive and sometimes lead to the loss of entire crop. In recent years, India and many countries around the world have experienced the devastating effect of the viral diseases, caused by nodavirus and extra small virus on production system, resulting in losses of several million dollars and identified as a major constraint to the rapid spread of this high-profit industry¹⁹¹⁻¹⁹³. Hence, knowledge of major diseases and the defence mechanism of freshwater prawn are crucial for sustainable development of scampi industry. Majority of disease conditions observed in cultured freshwater prawns are caused by a combination of pathogenic, nutritional, physiological and environmental factors. Proper management of water quality and well balanced diet will immensely help in reducing such infections. Furthermore, immunostimulants like β -glucan, lactoferrin etc, which build up protection against broad spectrum of pathogens, become increasingly important to enhance the immune status of freshwater prawn^{58, 118, 194} after realization of the side effects of antibiotics treatment and other chemotherapeutics. In cases, where disease outbreaks are cyclic and can be predicted, immunostimulants may be used in anticipation of events to elevate the defence mechanism and thus prevent further losses from disease. In addition, provision of

quarantine facility at the farm site, also helps to prevent the transfer of disease agents with live aquatic animal movements¹⁹⁵. Quarantine programmes that form part of the first line of defence against possible adverse effects resulting from the introduction or transfer of exotic fish and shellfish should be given importance.

References

1. New MB. Status of freshwater prawn farming. *Aqua Res* 1995; **26** : 1-54.
2. New MB. History and global status of freshwater prawn farming. In: New MB, Valenti WC, editors. *Freshwater Prawn Culture, the farming of *Macrobrachium rosenbergii**. Oxford, UK. Blackwell Science, 2000 pp 1-17
3. New MB. Freshwater prawn farming: global status, recent research and a glance at the future. *Aqua Res* 2005; **36** : 210-230.
4. FAO. Fisheries statistics aquaculture production 2005 Rome: 2007; **100/2** p. 1-90
5. Sahul Hameed AS, Yoganandhan K, Widada JS, Bonami JR. Studies on the occurrence of *Macrobrachium rosenbergii* nodavirus and extra small virus-like particles associated with white tail disease of *M. rosenbergii* in India by RT-PCR detection. *Aquaculture* 2004; **238** : 127-133.
6. Sahul Hameed AS, Yoganandhan K, Widada JS, Bonami JR. Experimental transmission and tissue tropism of *Macrobrachium rosenbergii* nodavirus (MrNV) and its associated extra small virus (XSV). *Dis Aquat Org* 2004; **62** : 191-196
7. Sahoo PK, Tripathy S, Mishra BK, Adhikari S, Das BK, Nandi S, Hari Babu P, Sarangi N, Ayyappan S. Is appendage deformity syndrome caused by *Macrobrachium rosenbergii* nodavirus? *Curr Sci* 2005; **88** : 1374-1375
8. Shekhar MS, Azad IS, Jithendran KP. RT-PCR and sequence analysis of *Macrobrachium rosenbergii* nodavirus: Indian isolate. *Aquaculture* 2006; **252** : 128-132.
9. Vijayan KK, Raj VS, Alavandi SV, Sekhar VT, Santiago TC. Incidence of white muscle disease, a viral like disease associated with mortalities in hatchery-reared postlarvae of the giant freshwater prawn *Macrobrachium rosenbergii* (de Man) from south-east coast of India. *Aqua Res* 2005; **36**: 311-316.
10. Arcier JM, Herman F, Lightner DV, Redman RM, Mari J, Bonami JR. A viral disease associated with mortalities in hatchery-reared postlarvae of the giant freshwater prawn *Macrobrachium rosenbergii*. *Dis Aquat Org* 1999; **38** : 177-181.
11. Qian D, Shi ZL, Chao Z, Liu W, Zhang SY, Bonami JR. Isolation and characteristics of nodavirus causing the whitish

- muscle disease of *Macrobrachium rosenbergii* *J Fish Sci China* 2003; **10** : 457-458
12. Qian D, Shi Z, Zhang S, Cao Z, Liu W, Li L, Xie Y, Cambournac I, Bonami JR. Extra small virus-like particles (XSV) and nodavirus associated with whitish muscle disease in the giant freshwater prawn, *Macrobrachium rosenbergii* *J Fish Dis* 2003; **26** : 521-527.
 13. Bonami JR, Shi Z, Qian D, Widada JS. White tail disease of the giant freshwater prawn, *Macrobrachium rosenbergii* separation of the associated virions and characterization of MrNV as a new type of nodavirus. *J Fish Dis* 2005; **28** : 23-31.
 14. Romestand B, Bonami JR. A sandwich enzyme linked immunosorbent assay (S-ELISA) for detection of MrNV in the giant freshwater prawn, *Macrobrachium rosenbergii* (de Man). *J Fish Dis* 2003; **26** : 71-75.
 15. Widada JS, Bonami JR. Characteristics of the monocistronic genome of extra small virus, a virus-like particle associated with *Macrobrachium rosenbergii* nodavirus: possible candidate for a new species of satellite virus *J Gen Virol* 2004; **85** : 643-646.
 16. Sudhakaran R, Musthaq SS, Haribabu P, Mukherjee SC, Gopal C, Hameed ASS. Experimental transmission of *Macrobrachium rosenbergii* nodavirus (MrL) and extra small virus (XSV) in three species of marine shrimp (*Penaeus indicus*, *Penaeus japonicus* and *Penaeus monodon*). *Aquaculture* 2006; **257** : 136-141.
 17. Qian D, Liu W, Jianxiang W, Yu L. Preparation of monoclonal antibody against *Macrobrachium rosenbergii* nodavirus and application of TAS-ELISA for virus diagnosis in post-larvae hatcheries in east China during 2000-2004. *Aquaculture* 2006; **261** : 1144-1150.
 18. Widada JS, Durand S, Cambournac I, Qian D, Shi Z, Dejonghe E, Richard V, Bonami JR. Genome-based detection methods of *Macrobrachium rosenbergii* nodavirus, a pathogen of the giant freshwater prawn, *Macrobrachium rosenbergii*: dot-blot, *in situ* hybridization and RT-PCR. *J Fish Dis* 2003; **26** : 583-590.
 19. Widada JS, Richard V, Shi Z, Qian D, Bonami JR. Dot-blot hybridization and RT-PCR detection of extra small virus (XSV) associated with white tail disease of prawn *Macrobrachium rosenbergii*. *Dis Aquat Org* 2004; **58** : 83-87.
 20. Yoganandhan K, Widada JS, Bonami JR, Hameed ASS. Simultaneous detection of *Macrobrachium rosenbergii* nodavirus and extra small virus by a single tube, one-step multiplex RT-PCR assay. *J Fish Dis* 2005; **28** : 65-69.
 21. Tripathy S, Sahoo PK, Kumari J, Mishra BK, Sarangi N, Ayyappan S. Multiplex RT-PCR detection and sequence comparison of viruses MrNV and XSV associated with white tail disease in *Macrobrachium rosenbergii*. *Aquaculture* 2006; **258** : 134-139.
 22. Anonymous. RT-PCR based diagnostic for *Macrobrachium rosenbergii* nodavirus. In: Sarangi N, Mohapatra BC, Dhir BL, editors. CIFA News 11. Bhubaneswar, India: Central Institute of Freshwater Aquaculture 2004 pp 3-4.
 23. Pillai D, Bonami JR, Widada JS. Rapid detection of *Macrobrachium rosenbergii* nodavirus (MrNV) and extra small virus (XSV), the pathogenic agents of white tail disease of *Macrobrachium rosenbergii* (de Man), by loop-mediated isothermal amplification. *J Fish Dis* 2006; **29** : 275-283.
 24. Bonami JR, Lightner DV. Unclassified viruses of crustacea. In: Adams JR, Bonami JR, editors. Atlas of Invertebrate Viruses Boca Raton, FL: CRC Press, 1991. pp. 597-622.
 25. Yang B, Song X-L, Huang J, Shi C-Y, Liu Q-H, Liu L. A single-step multiplex PCR for simultaneous detection of white spot syndrome virus and infectious hypodermal and haematopoietic necrosis virus in penaeid shrimp. *J Fish Dis* 2006; **29** : 301-305.
 26. Bonami JR, Trumper B, Mari J, Brehelin M, Lightner DV. Purification and characterization of the infectious hypodermal and haematopoietic necrosis virus of penaeid shrimps. *J Gen Virol* 1990; **71** : 2657-2664.
 27. Lightner DV, Redman RM, Bell TA. Infectious hypodermal and haematopoietic necrosis (IHHN), a newly recognised virus disease of penaeid shrimp. *J Invertebr Pathol* 1983; **42** : 62-70.
 28. Lightner DV, Redman RM, Bell TA, Brock JA. Detection of IHHN virus in *Penaeus stylirostris* and *P. vannamei* imported into Hawaii. *J World Maricult Soc* 1983; **14** : 212-225.
 29. Bell TA, Lightner DV. IHHN virus: Infectivity and pathogenicity studies in *Penaeus stylirostris* and *Penaeus vannamei*. *Aquaculture* 1984; **38** : 185-194.
 30. Kalagayan H, Godin D, Kanna R, Hagino G, Sweeney J, Wyban J, Brock J. IHHN virus as an etiological factor in runt-deformity syndrome (RDS) of juvenile *Penaeus vannamei* cultured in Hawaii. *J World Aquacult Soc* 1991; **22** : 235-243.
 31. Primavera JH, Quintio ET. Runt-deformity syndrome in cultured giant tiger prawn *Penaeus monodon*. *J Crust Biol* 2000; **20** : 796-802.
 32. Hsieh CY, Chuang PC, Chen LC, Tu C, Chien MS, Huang KC, Kao HF, Tung MC, Tsai SS. Infectious hypodermal and haematopoietic necrosis virus (IHHNV) infections in giant freshwater prawn, *Macrobrachium rosenbergii*. *Aquaculture* 2006; **258** : 73-79.
 33. Lo CF, Ho CH, Peng SE, Chen CH, Su HC, Chiu YL, Chang CF, Liu KF, Su MS, Wang CH, Kou GH. White spot syndrome baculovirus (WSBV) detected in cultured and captive shrimp, crabs and other arthropods. *Dis Aquat Org* 1996; **27** : 215-225.

34. Panchayuthapani D. A survey of shrimp diseases in India. In: Flegel TW, MacRae IH, editors. Diseases in Asian Aquaculture III Manila: Asian Fisheries Society, 1997. pp. 225-232
35. Chou HY, Huang CY, Lo CF, Kou GH. Studies on transmission of white spot syndrome associated baculovirus (WSBV) in *Penaeus monodon* and *P. japonicus* via waterborne contact and oral ingestion. *Aquaculture* 1998; **164** : 263-276.
36. Nunan LM, Poulos BT, Lightner DV. The detection of white spot syndrome virus (WSSV) and Yellow head virus (YHV) in imported commodity shrimp. *Aquaculture* 1998; **160** : 19-30.
37. Rajendran KV, Vijayan KK, Santiago TC, Krol RM. Experimental host range and histopathology of white spot syndrome virus (WSSV) infection in shrimp, prawns, crabs and lobsters from India. *J Fish Dis* 1999; **22** : 183-191.
38. Peng SE, Lo CF, Ho CH, Chang CF, Kou GH. Detection of white spot baculovirus (WSBV) in giant freshwater prawn, *Macrobrachium rosenbergii*, using polymerase chain reaction. *Aquaculture* 1998; **164** : 253-262.
39. Sahul Hameed AS, Charles MX, Anilkumar M. Tolerance of *Macrobrachium rosenbergii* to white spot syndrome virus. *Aquaculture* 2000; **183** : 207-213.
40. Kiran RBP, Rajendran KV, Jung SO, Oh MJ. Experimental susceptibility of different life-stages of the giant freshwater prawn, *M. rosenbergii* (de Man), to white spot syndrome virus (WSSV) *J Fish Dis* 2002; **25** : 201-207.
41. Tung CW, Wang CS, Chen SN. Histological and electron microscopic study on *Macrobrachium* muscle virus (MMV) infection in the giant freshwater prawn, *Macrobrachium rosenbergii* (de Man) culture in Taiwan *J Fish Dis* 1999; **22** : 319-323.
42. Anderson IG, Law AT, Shariff M, Nash G. A parvo-like virus in the giant freshwater prawn, *Macrobrachium rosenbergii* *J Invertebr Pathol* 1990; **55** : 447-449.
43. Lightner DV. A Handbook of Pathology and Diagnostic Procedures for Diseases of Penaeid Shrimp. Baton Rouge. World Aquaculture Society, 1996.
44. Lalitha KV, Surendran PK. Bacterial microflora associated with farmed freshwater prawn *Macrobrachium rosenbergii* (de Man) and the aquaculture environment. *Aqua Res* 2004; **35** : 629-635.
45. Fujioka R, Greco S. Enumeration of *Vibrio* sp. in marine, brackish and freshwater systems used in aquaculture. Annual Meeting of the American Society for Microbiology, 1984, Washington.
46. Baticados MCL, Cruz-Lacierda ER, de la Cruz MC, Duremdz-Fernandez RC, Gacutan RQ, Lavilla-Pitogo CR, Lio-Po GD. Diseases of penaeid shrimps in the Philippines Tigbauan, Iloilo: Aquaculture Department, SEAFDEC, 1990.
47. Jayaprakash NS, Pai SS, Anas A, Preetha R, Philip R, Singh ISB. A marine bacterium, *Micrococcus* MCCB 104, antagonistic to vibrios in prawn larval rearing systems *Dis Aquat Org* 2005; **68** : 39-45.
48. Jayaprakash NS, Kumar VJR, Philip R, Singh ISB. Vibriosis associated with *Macrobrachium rosenbergii* (de Man, 1879) larvae from three hatcheries on the Indian southwest coast. *Aqua Res* 2006; **37** : 351-358
49. Jayaprakash NS, Pai SS, Philip R, Singh ISB. Isolation of a pathogenic strain of *Vibrio alginolyticus* from necrotic larvae of *Macrobrachium rosenbergii* (de Man) *J Fish Dis* 2006; **29** : 187-191.
50. Sahul Hameed AS, Rahaman KH, Alagan A, Yoganandhan K. Antibiotic resistance in bacteria isolated from hatchery-reared larvae and post-larvae of *Macrobrachium rosenbergii* *Aquaculture* 2003; **217** : 39-48.
51. Takahashi Y, Shimoyama Y, Momoyama K. Pathogenicity and characteristics of *Vibrio* sp. isolated from diseased post larvae of Kuruma prawn, *Penaeus japonicus* Beta. *J Shimonoseki Univ Fish* 1985; **32** : 23-31.
52. Anas A, Paul S, Jayaprakash NS, Philip R, Singh ISB. Antimicrobial activity of chitosan against vibrios from freshwater prawn *Macrobrachium rosenbergii* larval rearing systems. *Dis Aquat Org* 2005; **67** : 177-179
53. Tonguthai K. Diseases of the freshwater prawn *Macrobrachium rosenbergii* in Thailand. In: Shariff IM, Subasinghe RP, Arthur JR, editors. Diseases in Asian Aquaculture. Manila: Asian Fisheries Society, 1992. pp. 89-95.
54. Tonguthai K. Diseases of the freshwater prawn, *Macrobrachium rosenbergii*. *AAHRI Newsletter* 1997; **4** : 1-4.
55. Karunasagar I, Pai R, Malathi GR, Karunasagar I. Mass mortality of *Penaeus monodon* larvae due to antibiotic resistance *Vibrio harveyi* infection. *Aquaculture* 1994; **128** : 203-209.
56. Tendencia EA, de la Pena LD. Antibiotic resistance of bacteria from shrimp ponds. *Aquaculture* 2001; **195** : 193-204.
57. Gomez-Gil B, Roque A, Turnbull JF. The use and selection of probiotic bacteria for use in the culture of larval aquatic organisms. *Aquaculture* 2000; **191** : 259-270.
58. Misra CK, Das BK, Pradhan J, Pattnaik P, Sethi S, Mukherjee SC. Changes in lysosomal enzyme activity and protection against *Vibrio* infection in *Macrobrachium rosenbergii* (de Man) post larvae after bath immunostimulation with β -glucan. *Fish Shellfish Immunol* 2004; **17** : 389-395.
59. Sahoo PK, Mukherjee SC. Influence of the immunostimulant, chitosan on the immune responses of

- healthy and cortisol treated rohu (*Labeo rohita*). *J Aqua Trop* 1999; **14** : 209-215.
60. Jayasree L, Janakiram P, Madhavi R. Shell disease in the freshwater prawn *Macrobrachium rosenbergii* (de Man): Aetiology, pathogenicity and antibiotic sensitivity. *J Aqua Trop* 1999; **14** : 289-298.
 61. El-Gamal AA, Alderman DJ, Rodgers CJ, Polglase JL, Macintosh D. A scanning electron microscope study of oxolinic acid treatment of burn spot lesions of *Macrobrachium rosenbergii*. *Aquaculture* 1986; **52** : 157-171.
 62. Sung HH, Hwang SF, Tasi FM. Responses of giant freshwater prawn (*Macrobrachium rosenbergii*) to challenge by two strains of *Aeromonas* spp. *J Invertebr Pathol* 2000; **76** : 278-284.
 63. Johnson SK, Bueno SLS. Health Management. In: New MB, Valenti WC, editors. *Freshwater Prawn Culture, The farming of Macrobrachium rosenbergii*. London: Blackwell Science, 2000. pp. 239-258.
 64. Brock JA. Diseases and husbandry problems of cultured *Macrobrachium rosenbergii*. In: Sindermann CJ, Lightner DV, editors. *Disease, Diagnosis and Control in North American Marine Aquaculture*. Second Edition, Amsterdam: Elsevier Scientific Publishing, 1988. pp. 134-180.
 65. Hipolito M, Baldassi L, Pires DC, Lombardi JV. Prevalencia bacteriana em necrose de camarao de agua doce (*Macrobrachium rosenbergii*, Decapoda, Palaemonidae). *Boletim do Instituto de Pesca* 1996; **23** : 13-20.
 66. Martinez LE, Molianres AM, Villanueva J, Conroy DA. Preliminary observations on the application of nifurpirinol for the control of potential disease problems in *Macrobrachium acanthurus*. In: New MB, editor. *Giant Prawn Farming, Developments in Aquaculture and Fisheries Science Vol. 10*. Amsterdam: Elsevier Scientific Publishing, 1982. pp. 285-289.
 67. Brock JA. A synopsis of pathology, diseases and production problems of cultured *Macrobrachium*, with an emphasis on experiences in Hawaiian prawn farming. In: McVey JP, editor. *CRC Handbook of Mariculture*, Second Edition, Vol. I. Crustacean Aquaculture. Boca Raton, FL: CRC Press, 1993. pp. 361-391.
 68. Delves-Broughton J, Poupard CW. Disease problems of prawns in recirculation systems in the U. K. *Aquaculture* 1976; **7** : 201-217.
 69. Aquacop. Observations on Diseases of Crustacean Cultures in Polynesia.: Proceedings of the 8th Annual Meeting of the World Mariculture Society. 1977, 9-13 January. Costa Rica.
 70. Le Moullac G, Soyez C, Saulnier D, Ansquer D, Avarre JC, Levy P. Effect of hypoxia stress on the immune response and the resistance to vibriosis of the shrimp *Penaeus stylirostris*. *Fish Shellfish Immunol* 1998; **8** : 621-629.
 71. Bueno SL de S, Gastelu JC. Doencas em camaroes de agua doce. In: Valenti WC, editor. *Carcinicultura de Agua Doce: Tecnologia para a Producao de Camaroes*, Fundacao de Amparo a Pesquisa do Estado De Sao Paulo (FAPESP), Brasilia: Sao Paulo and Instituto Brasileiro do Meio Ambiente e dos Recursos Naturais Renovaveis, 1998. pp. 309-339.
 72. Le Bitoux JF. Bacterial necrosis of *Macrobrachium* larvae. In: Sindermann CJ, editor. *Disease Diagnosis and Control in North American Marine Aquaculture*. Amsterdam: Elsevier Scientific Publishing, 1988. pp. 140-141.
 73. Brock JA. Disease and pathology considerations for *Macrobrachium* culture. In: Chavez CJ, editor. *The Aquaculture of Shrimp, Prawn and Crawfish in the World: Basics and Technologies*. Tokyo: Midori Shobo, 1990. pp. 1-226.
 74. Brock JA, Ankagawa LK, Shimojo RJ. Infection of cultured freshwater prawn *Macrobrachium rosenbergii* De Man (Crustacea: Decapoda) by *Mycobacterium* spp., Runyan Group II. *J Fish Dis* 1986; **9** : 319-324.
 75. Cheng W, Chen JC. Isolation and characterization of an *Enterococcus*-like bacterium causing muscle necrosis and mortality in *Macrobrachium rosenbergii* in Taiwan. *Dis Aquat Org* 1998; **34** : 93-101.
 76. Chen SC, Lin YD, Liaw LL, Wang PC. *Lactococcus garvieae* infection in the giant freshwater prawn *Macrobrachium rosenbergii* confirmed by polymerase chain reaction and 16s rDNA sequencing. *Dis Aquat Org* 2001; **45** : 45-52.
 77. Cheng W, Chen JC. *Enterococcus*-like infections in *Macrobrachium rosenbergii* are exacerbated by high pH and temperature but reduced by low salinity. *Dis Aquat Org* 1998; **34** : 103-108.
 78. Cohen D, Issar G. Rickettsial disease of *Macrobrachium rosenbergii* larvae: gross signs, diagnosis and treatment. *World Aquaculture*, 1990, 10-14 June, Halifax, Nova Scotia, Canada.
 79. Da Silva AN, Canuto e Silva JN, Cohen D, Issar G. A new rickettsial disease in *Macrobrachium rosenbergii* larvae: gross signs, cause, diagnosis and treatment. In: Martins MMR, Correia ES, Cavalheiro JM, editors. *Anais do III Simposio Brasileiro sobre Cultivo de Camarao*, 15-Joao Pessoa, Vol. 2: Joao Pessoa: Camarao de Agua Doce e Outros, MCR Aquaculture, 20 outubro, 1989. 317-337.
 80. Canuto e Silva JN, da Silva AN, Cohen D. The protective role of lime (CaO) and the adverse effect of *Artemia* in *Macrobrachium rosenbergii* hatchery under infection with *Rickettsia* sp. In: Martins MMR, Correia ES, Cavalheiro JM, editors. *Anais do III Simposio Brasileiro sobre Cultivo de Camarao*, Joao Pessoa, Vol. 2: Joao Pessoa: Camarao de Agua Doce e Outros, MCR Aquaculture, 20 outubro, 1989. 289-306.

81. Gomes NA, Coelho PA, Upadhyay HP. Presença de fungos em cultivos de larvas de camarão (*Macrobrachium rosenbergii* de Man) Anais do V Congresso Brasileiro de Engenharia de Pesca, Fortaleza, Associação dos Engenheiros de Pesca do Estado do Ceará, 1987, 26-31 julho, Fortaleza.
82. Lightner DV. Fungus (*Fusarium*) disease of juvenile and adult penaeid shrimp. In: Sindermann C, Lightner D, editor. Disease Diagnosis and Control in North American Marine Aquaculture, Amsterdam: Elsevier Scientific Publishing, 1988 64-69.
83. Lu CC, Tang FJ, Yoichiro U, Kuo GH, Chen SN. Yeast infection in prawns (*Macrobrachium rosenbergii* De Man) in Taiwan. *Acta Zool Taiwan* 1997; **8** : 33-45.
84. Su HY, Wang PC, Lien YY, Tsai MA, Liu SS, Wu KC, Hsieh CH, Chen SC. Upregulation of actin-like gene expression in giant freshwater prawns *Macrobrachium rosenbergii* infected with *Metschnikowia bicuspidata* *Dis Aquat Org* 2005; **66** : 175-180.
85. Lu CC, Tang KFJ, Chen SN. Identification and genetic characterization of yeasts isolated from freshwater prawns, *Macrobrachium rosenbergii* de Man, in Taiwan *J Fish Dis* 1998, **21** : 185-192.
86. Chien CY. Preliminary survey on the saprolegniaceous fungi parasites of freshwater shrimps (Crustaceans) in Taiwan. *Proc Nat Sci Council* 1976; **9** : 39-47.
87. Chen SN. Current status of shrimp aquaculture in Taiwan. Proceedings of the Special Session on Shrimp Farming 1995, 1-4 February, San Diego, Baton Rouge
88. Smith TIJ, Sandifer PA, Manzi JJ. Epibionts of pond-reared adult Malaysian prawns, *Macrobrachium rosenbergii* (de Man), in South Carolina. *Aquaculture* 1979; **16** : 299-308.
89. Johnson SK. Diseases of *Macrobrachium*. In: New MB, editor. Giant Prawn Farming, Developments in Aquaculture and Fisheries Science Vol 10. Amsterdam: Elsevier Scientific Publishing, 1982 269-277.
90. Roegge MA, Rutledge WP, Guest WC. Chemical control of *Zoothamnium* sp. on larval *Macrobrachium acanthurus* *Aquaculture* 1977; **12** : 137-140.
91. Sindermann CJ. Disease and disease control in *Macrobrachium* culture. In: Hanson JA, Goodwin HL editors. Freshwater Prawn Farming in the Western Hemisphere. Stroudsburg. Dowden Hutchinson and Ros Inc, 1977. 210-219.
92. Mariappan P, Balasundaram C, Trilles JP. Infection of the isopod *Tachaea spongillicola* on freshwater prawns *Macrobrachium* spp. in southern India. *Dis Aquat Org* 2003; **55** : 259-260.
93. Truesdale FM, Mermilliod WJ. Some observations on the host-parasite relationship of *Macrobrachium ohione* (Smith) (Decapoda, Palaemonidae) and *Probopyrus bithynis* Richardson (Isopoda, Bopyridae). *Crustaceana* 1977, **32** : 216-220
94. Johnson SK. Handbook of Crawfish and Freshwater Shrimp Diseases Texas: Texas A & M University Sea Grant College Program, 1978.
95. Nash G. Trematode metacercarial infection of cultured giant freshwater prawns, *Macrobrachium rosenbergii* *J Invertebr Pathol* 1989; **53** : 124-127
96. Kumar AR, Rao GV, Rao KRS Appendage deformity syndrome-a nutritional disease of *Macrobrachium rosenbergii*. *Dis Aquat Org* 2004; **59** : 75-78.
97. Saurabh S, Sahoo PK. Appendage deformity syndrome (ADS): An emerging disease of giant freshwater prawn, *Macrobrachium rosenbergii* *Aqua Int* 2007; **14** : p 20.
98. Pillai D, Nair CM, Salin KR, Marques A, Widada JS, Bonami JR. Gross signs and histopathology of branchiostegal blister disease (balloon disease): an idiopathic disease of farmed *Macrobrachium rosenbergii* (de Man). *J Fish Dis* 2005; **28** : 473-478.
99. Salin KR, Nair CM Emerging diseases of giant freshwater prawn in India: a potential threat to sustainability. International Symposium on Freshwater Prawns, 2003, 20-23 August, Kochi, India.
100. Akiyama DM, Brock JA, Haley SR. Idiopathic muscle necrosis in the cultured freshwater prawn, *Macrobrachium rosenbergii*. *Vet Med/Small Anim Clinic* 1982; **77** : 1119-1121.
101. Nash G, Chinabut S, Limsuwan C Idiopathic muscle necrosis in the freshwater prawn, *Macrobrachium rosenbergii* de Man, cultured in Thailand. *J Fish Dis* 1987; **10** : 109-120
102. Anderson IG, Nash G, Shariff M. Mass larval mortalities in the giant freshwater prawn, *M. rosenbergii* (de Man), cultured in Malaysian modified static greenwater systems *J Fish Dis* 1990; **13** : 127-134.
103. Akita G, Ankamura R, Brock J, Miyamoto G, Fujimoto M, Oishi F, Onizuka D, Sumikawa D. Epizootiologic study of mid-cycle disease of larval *Macrobrachium rosenbergii* *J World Mari Soc* 1981; **12** : 223-230.
104. Kanaujia DR, Das BK, Mohanty AN. Mass larval mortalities in Indian river prawn, *Macrobrachium malcolmsonii* under hatchery conditions and their control by application of antibiotics. *J Aqua Trop* 1998; **13** : 171-179.
105. Wickens JF. The tolerance of warm water prawns to recirculated water. *Aquaculture* 1976; **9** : 19-37.
106. Armstrong DA, Stephenson MJ, Knight AW. Acute toxicity of nitrite to larvae of the giant Malaysian prawn, *Macrobrachium rosenbergii* *Aquaculture* 1976; **9** : 39-46

107. Sahoo PK. Defence mechanism in shellfish. In: Mishra BK, Swain P, Sahoo PK, Das BK, Sarangi N, editors. Summer school on disease diagnosis and health management of freshwater fish and shellfish. Bhubaneswar, India: Central Institute of Freshwater Aquaculture, 2004. 74-78.
108. Tsing A, Arcier JM, Brehelin M. Haemocytes of penaeids and palaemonid shrimps: morphology, cytochemistry and hemograms *J Invertebr Pathol* 1989; **53** : 64-77.
109. Holmblad T, Soderhall K. Cell adhesion molecules and antioxidative enzymes in a crustacean, possible role in immunity. *Aquaculture* 1999; **172** : 111-123.
110. Smith VJ, Chisholm JRS. Non cellular immunity in crustaceans. *Fish Shellfish Immunol* 1992; **2** : 1-31.
111. Smith VJ, Chisholm JRS. Antimicrobial proteins in crustaceans. *Adv Exp Med Biol* 2001; **484** : 95-112.
112. Hose JE, Martin GG, Gerard AS. A decapod hemocyte classification scheme integrating morphology, cytochemistry, and function. *Biol Bull* 1990; **178** : 33-45.
113. Smith VJ, Brown JH, Hauton C. Immunostimulation in crustaceans: does it really protect against infection? *Fish Shellfish Immunol* 2003; **15** : 71-90.
114. Bachere E, Miahle E, Rodriguez J. Identification of defence effectors in the haemolymph of crustaceans with particular reference to the shrimp *Penaeus japonicus* (Bate): prospects and application. *Fish Shellfish Immunol* 1995; **5** : 597-612.
115. Itami T, Asano M, Tokushige K, Kubono K, Nakagawa A, Takeno N *et al.* Enhancement of disease resistance of kuruma shrimp, *Penaeus japonicus*, after oral administration of peptidoglycan derived from *Bifidobacterium thermophilum*. *Aquaculture* 1998; **164** : 277-288.
116. Sung HH, Kuo PA, Kao WY. Effect of lipopolysaccharide on *in vitro* phagocytosis by hemocytes from giant freshwater prawn (*Macrobrachium rosenbergii*). *Fish Pathol* 2000; **35** : 109-116.
117. Sierra C, Guevara J, Lasscurain R, Perez A, Agundis C, Zenteno E. *et al.* Sialylation is modulated through maturation in hemocytes from *Macrobrachium rosenbergii*. *Comp Biochem Physiol* 2001; **130C** : 179-189.
118. Chand RK, Sahoo PK, Kumari J, Pillai BR, Mishra BK. Dietary administration of bovine lactoferrin influences the immune ability of the giant freshwater prawn *Macrobrachium rosenbergii* (de Man) and its resistance against *Aeromonas hydrophila* infection and nitrite stress. *Fish Shellfish Immunol* 2006; **21** : 119-129.
119. Johansson MW, Söderhäll K. A cell adhesion factor from crayfish haemocytes has degranulating activity towards crayfish granular cells. *Insect Biochem* 1989; **19** : 183-190.
120. Yoshino TP. Surface membrane components of circulating invertebrate blood cells and their role in internal defence. In: Brehelin M, editor. Immunity in Invertebrates: Cells, Molecules and Defence Reactions. Berlin: Springer-Verlag Press, 1986. 12-24.
121. Hsu JP, Huang C, Liao CM, Hsuan SL, Hung HH, Chien MS. Engulfed pathogen-induced apoptosis in haemocytes of giant freshwater prawn, *Macrobrachium rosenbergii* *J Fish Dis* 2005; **28** : 729-735.
122. Sindermann CJ. Internal defenses of crustacea: a review. *Fish Bull* 1971; **69** : 455-489.
123. Soderhall K, Cerenius L. Crustacean immunity. *Ann Rev Fish Dis* 1992; **2** : 3-23.
124. Bauchau AG. Crustaceans. In: Ratcliffe NA, Rowley AF, editors. Invertebrate Blood Cells. London. Academic Press Inc, 1981. 385-420.
125. Lee SY, Soderhall K. Early events in crustacean immunity *Fish Shellfish Immunol* 2002; **12** : 421-437.
126. Roch P. Defence mechanisms and disease prevention in farmed marine invertebrates. *Aquaculture* 1999; **172** : 125-145.
127. Song YL, Hsieh YT. Immunostimulation of tiger shrimp (*Penaeus monodon*) hemocytes for generation of microbicidal substances: analysis of reactive oxygen species. *Dev Comp Immunol* 1994; **18** : 201-209.
128. Bell KL, Smith VJ. *In vitro* superoxide production by hyaline cells of the shore crab *Carcinus maenas* (L.). *Dev Comp Immunol* 1993; **17** : 211-219.
129. Liu CH, Yeh ST, Cheng SY, Chen JC. The immune response of the white shrimp *Litopenaeus vannamei* and its susceptibility to *Vibrio* infection in relation with the moult cycle. *Fish Shellfish Immunol* 2004; **16** : 151-161.
130. Muñoz M, Cedeño R, Rodríguez J, Van der Knapp WPW, Mialhe E, Bachère E. Measurement of reactive oxygen intermediate production in haemocytes of the penaeid shrimp, *Penaeus vannamei*. *Aquaculture* 2000; **191** : 89-107.
131. Tseng IT, Chen JC. The immune response of white shrimp *Litopenaeus vannamei* and its susceptibility to *Vibrio alginolyticus* under nitrite stress. *Fish Shellfish Immunol* 2004; **17** : 325-333.
132. Acharya S, Mohanty J, Sahoo PK. Humoral defence factors in Indian river prawn, *Macrobrachium malcolmsonii* *Fish Shellfish Immunol* 2004; **17** : 137-147.
133. Chand RK, Sahoo PK. Effect of nitrite on the immune response of freshwater prawn *Macrobrachium malcolmsonii* and its susceptibility to *Aeromonas hydrophila*. *Aquaculture* 2006; **258** : 150-156.
134. Funte M De la, Victor VM. Anti-oxidants as modulators of immune function. *Immunol Cell Biol* 2000; **78** : 49-54.

135. Campa-Córdova AI, Hernández-Saavedra NY, Philippis RDe, Ascencio F. Generation of superoxide anion and SOD activity in haemocytes and muscle of American white shrimp (*Litopenaeus vannamei*) as a response to β -glucan and sulphated polysaccharide. *Fish Shellfish Immunol* 2002; **12**: 353-366.
136. Cheng W, Tung YH, Chiou TT, Chen JC. Molecular cloning and characterisation of mitochondrial manganese superoxide dismutase (mtMnSOD) from the giant freshwater prawn *Macrobrachium rosenbergii*. *Fish Shellfish Immunol* 2006; **21**: 453-466.
137. Fridovich I. Superoxide radical and superoxide dismutases. *Ann Rev Biochem* 1995; **64**: 97-112.
138. Brouwer M, Brouwer TH, Grater W, Brown-Peterson NB. Replacement of a cytosolic copper/zinc superoxide dismutase by a novel cytosolic manganese superoxide dismutase in crustaceans that use copper (haemocyanin) for oxygen transport. *Biochem J* 2003; **374**: 219-228.
139. Smith MW, Doolittle EF. A comparison of evolutionary rates of the two major kinds of superoxide dismutase. *J Mol Evol* 1992; **34**: 175-184.
140. Gross PS, Bartlett TC, Browdy CL, Chapman RW, Warr GW. Immune gene discovery by expressed sequence tag analysis of hemocytes and hepatopancreas in the Pacific white shrimp, *Litopenaeus vannamei*, and the Atlantic white shrimp, *L. setiferus*. *Dev Comp Immunol* 2001; **25**: 565-577.
141. Brouwer M, Brouwer TH, Grater W, Enghild JJ, Thøgersen IB. The paradigm that all oxygen-respiring eukaryotes have cytosolic Cu/Zn-superoxide dismutase and that Mn-superoxide dismutase is localized to the mitochondrion does not apply to a large group of marine arthropods. *Biochem* 1997; **36**: 13381-13388.
142. Cheng W, Tung YH, Liu CH, Chen JC. Molecular cloning and characterisation of cytosolic manganese superoxide dismutase (cytMn-SOD) from the giant freshwater prawn *Macrobrachium rosenbergii*. *Fish Shellfish Immunol* 2006; **20**: 438-449.
143. Cheng W, Tung YH, Liu CH, Chen JC. Molecular cloning and characterisation of copper/zinc superoxide dismutase (Cu, Zn-SOD) from the giant freshwater prawn *Macrobrachium rosenbergii*. *Fish Shellfish Immunol* 2006; **21**: 102-112.
144. Johansson MW, Söderhäll K. Cellular immunity in crustaceans and the proPO system. *Parasitol Today* 1989; **5**: 171-176.
145. Sung HH, Chang HJ, Her CH, Chang JC, Song YL. Phenoloxidase activity of haemocytes derived from *Penaeus monodon* and *Macrobrachium rosenbergii*. *J Invertebr Pathol* 1998; **71**: 26-33.
146. Unestam T, Söderhäll K. Soluble fragments from fungal cell walls elicit defence reactions in crayfish. *Nature* 1977; **267**: 45-46.
147. Söderhäll K, Unestam T. Activation of serum prophenoloxidase in arthropod immunity. The specificity of cell wall glucan activation and activation by purified fungal glycoproteins of crayfish phenoloxidase. *Can J Microbiol* 1979; **25**: 406-414.
148. Smith VJ, Söderhäll K, Hamilton M. β -1,3-glucan induced cellular defence reaction in the shore crab, *Carcinus maenas*. *Comp Biochem Physiol* 1984; **77A**: 636-639.
149. Ashida M, Ishizaki Y, Iwahana H. Activation of prophenoloxidase by bacterial cell walls or β -1, 3-glucan in plasma of the silkworm, *Bombyx mori*. *Biochem Biophys Res Commun* 1983; **113**: 562-568.
150. Söderhäll K, Hall L. Lipopolysaccharide-induced activation of prophenoloxidase activating system in crayfish haemocyte lysate. *Biochem Biophys Acta* 1984; **797**: 99-104.
151. Ashida M, Söderhäll K. The prophenoloxidase activating system in crayfish. *Comp Biochem Physiol* 1984; **77B**: 21-26.
152. Dularay B, Lackie AM. Haemocytic encapsulation and the prophenoloxidase-activation pathway in the locust *Schistocerca gregaria*. *Insect Biochem* 1985; **15**: 827-834.
153. Leonard C, Söderhäll K, Ratcliffe NA. Studies on prophenoloxidase and protease activity of *Blaberus craniifer*. *Insect Biochem* 1985; **15**: 803-810.
154. Sugumaran M, Nellaiappan K. Lysolecithin-A potent activator of prophenoloxidase from the hemolymph of the lobster, *Homarus americanus*. *Biochem Biophys Res Commun* 1991; **176**: 1371-1376.
155. Aspán A, Sturtevant J, Smith VJ, Söderhäll K. Purification and characterization of a prophenoloxidase activating enzyme from crayfish blood cells. *Insect Biochem* 1990; **20**: 709-718.
156. Nappi M, Vass E. Melanogenesis and the generation of cytotoxic molecules during insect cellular immune reactions. *Pigm Cell Res* 1993; **6**: 117-126.
157. Hernandez-Lopez J, Gollas-Galvan TS, Vargas-Albores F. Activation of the prophenoloxidase system of the brown shrimp (*Penaeus californiensis* Holmes). *Comp Biochem Physiol* 1996; **113C**: 61-66.
158. Perazzolo LM, Barracco MA. The prophenoloxidase activating system of the shrimp, *Penaeus paulensis* and associated factors. *Dev Comp Immunol* 1997; **21**: 385-395.
159. Sritunyalucksana K, Sithisarn P, Withayachumnarnkul B, Flegel TW. Activation of prophenoloxidase, agglutinin and antibacterial activity in haemolymph of the black tiger prawn, *Penaeus monodon*, by immunostimulants. *Fish Shellfish Immunol* 1999; **9**: 21-30.

160. Albores VF, Plascencia YG. Beta glucan binding protein and its role in shrimp immune response. *Aquaculture* 2000; **191** : 13-21.
161. Lee SY, Wang R, Söderhäll K. A lipopolysaccharide- and beta-1,3-glucan-binding protein from hemocytes of the freshwater crayfish *Pacifastacus leniusculus*. Purification, characterization, and cDNA cloning. *J Bio Chem* 2000; **275** : 1337-1343.
162. Johansson MW, Lind MI, Holmblad T, Thörnqvist PO, Söderhäll K. Peroxinectin, a novel cell adhesion protein from crayfish blood. *Biochem Biophys Res Commun* 1995; **216** : 1079-1087.
163. Sritunyalucksana K, Wongsuebsantati K, Johansson MW, Söderhäll K. Peroxinectin, a cell adhesive protein associated with the proPO system from the black tiger shrimp, *Penaeus monodon*. *Dev Comp Immunol* 2001; **25** : 353-363.
164. Liu CH, Cheng W, Kuo CM, Chen JC. Molecular cloning and characterisation of a cell adhesion molecule, peroxinectin from the white shrimp *Litopenaeus vannamei*. *Fish Shellfish Immunol* 2003; **17** : 13-26.
165. Rattanachai A, Hirono I, Ohira T, Takahashi Y, Takashi A. Molecular cloning and expression analysis of $\alpha 2$ -macroglobulin in the kuruma shrimp, *Marsupenaeus japonicus*. *Fish Shellfish Immunol* 2004; **16** : 599-611.
166. Lee SY, Söderhäll K. Characterization of a pattern recognition protein, a masquerade-like protein, in the freshwater crayfish *Pacifastacus leniusculus*. *J Immunol* 2001; **166** : 7319-7326.
167. Wang R, Lee SY, Cerenius L, Söderhäll K. Properties of the prophenoloxidase activating enzyme of the freshwater crayfish, *Pacifastacus leniusculus*. *Eur J Biochem* 2001; **268** : 895-902.
168. Johansson MW, Söderhäll K. Isolation and purification of a cell adhesion factor from crayfish blood cells. *J Cell Biol* 1988; **106** : 1795-1803.
169. Johansson MW. Cell adhesion molecules in invertebrate immunity. *Dev Comp Immunol* 1999; **23** : 303-315.
170. Liu CH, Cheng W, Chen JC. The peroxinectin of white shrimp *Litopenaeus vannamei* is synthesised in the semi-granular and granular cells and its transcription is up-regulated with *Vibrio alginolyticus* infection. *Fish Shellfish Immunol* 2005; **18** : 431-444.
171. Kobayashi M, Johansson MW, Söderhäll K. The 76 kDa cell-adhesion factor from crayfish haemocytes promotes encapsulation *in vitro*. *Cell Tissue Res* 1990; **260** : 113-118.
172. Thörnqvist PO, Johansson MW, Söderhäll K. Opsonic activity of cell adhesion proteins and β -1,3-glucan-binding proteins from two crustaceans. *Dev Comp Immunol* 1994; **18** : 3-12.
173. Hsu PI, Liu CH, Tseng DY, Lee PP, Cheng W. Molecular cloning and characterisation of peroxinectin, a cell adhesion molecule, from the giant freshwater prawn *Macrobrachium rosenbergii*. *Fish Shellfish Immunol* 2006; **21** : 1-10.
174. Karunasagar I, Karunasagar I. Vaccines and immunostimulants for sustainable aquaculture. In: Karunasagar I, Karunasagar I, Reilly A, editors. *Aquaculture and Biotechnology*. New Delhi: Oxford and IBH Publishing Co. Pvt. Ltd, 1999. pp. 33-46.
175. Arason GJ. Lectins as defence molecules in vertebrates and invertebrates. *Fish Shellfish Immunol* 1996; **6** : 277-289.
176. Vazquez L, Jaramillo L, Lascrain R, Cooper EL, Rosas P, Zenteno E. Bacterial agglutination by the sialic acid specific serum lectin from *Macrobrachium rosenbergii*. *Comp Biochem Physiol* 1996; **113B** : 355-359.
177. Zenteno R, Vazquez L, Sierra C, Pereyra A, Slomiany MC, Bouquelet S, Zenteno E. Chemical characterization of the lectin from the freshwater prawn *Macrobrachium rosenbergii* (de Man) by MALDI-TOF. *Comp Biochem Physiol* 2000; **127B** : 243-250.
178. Agundis C, Pereyra A, Zenteno R, Brassart C, Sierra C, Vazquez L, Zenteno E. Quantification of lectin in freshwater prawn (*Macrobrachium rosenbergii*) hemolymph by ELISA. *Comp Biochem Physiol* 2000; **127B** : 165-172.
179. Pereyra A, Zenteno R, Vazquez L, Cairo SM, Rodriguez A, Hernandez GM, Zenteno E, Agundis C. Characterization of lectin aggregates in the hemolymph of freshwater prawn *Macrobrachium rosenbergii*. *Biochimica et Biophysica Acta* 2004; **1673** : 122-130.
180. Sierra C, Lascrain R, Pereyra A, Guevara J, Martinez G, Agundis C, Zenteno E, Vazquez L. Participation of serum and membrane lectins on the oxidative burst regulation in *Macrobrachium rosenbergii* hemocytes. *Dev Comp Immunol* 2005; **29** : 113-121.
181. Soria F, Sierra C, Bouquelet S, Brassart C, Agundis C, Zenteno E, Vazquez L. The effect of sugars and free amino acids from the freshwater prawn *Macrobrachium rosenbergii* hemolymph on lectin activity and on oxidative burst. *Comp Biochem Physiol* 2006; **142C** : 212-219.
182. Renwrandt L. Lectins in molluscs and arthropods: their occurrence, origin and roles in immunity. *Symp Zool Soc Lond* 1986; **56** : 81-93.
183. Cooper EL, Rinkevich B, Uhlenbruck G, Valembois P. Invertebrate immunity: another viewpoint. *Scand J Immunol* 1992; **35** : 247-266.
184. Goldstein IJ, Hughes RC, Monsigny M, Osawa T, Sharon N. What should be called a lectin?. *Nature* 1980; **66** : 285-286.
185. Sharon N. Surface carbohydrates and surface lectins are recognition determinants in phagocytosis. *Immunol Today* 1984; **5** : 1-5.

186. Nesser R, Koellreutter B, Wuersch P. Oligomannoside type glycopeptides inhibiting adhesion of *Escherichia coli* strains mediated by type 1 pili, preparation of potent inhibitors from plant glycopeptides. *Infect Immun* 1986; **58** : 428-436.
187. Vazquez L, Masso F, Rosas P, Montaño LF, Zenteno E. Purification and characterization of a lectin from *Macrobrachium rosenbergii* (Crustacea, Decapoda) hemolymph *Comp Biochem Physiol B* 1993; **105** : 617-623.
188. Vazquez L, Lanz H, Montaño LF, Zenteno E. Biological activity of the lectin from *Macrobrachium rosenbergii*. In: Van Driessche E, Bog-Hansen TC, editors. *Lectins, Biology, Biochemistry, Clinical Chemistry*. Hellerup, Denmark: Textop, 1994, vol. **10**, 261-265.
189. Guevara J, Espinosa B, Zenteno E, Vazquez L, Luna J, Perry G, Mena R. Altered glycosylation pattern of proteins in Alzheimer's disease. *J Neuropathol Exp Neurol* 1998; **57** : 905-914.
190. FAO. Asia regional technical guidelines on health management for the responsible movement of live aquatic animal and the Beijing consensus and implementation strategy. FAO Fisheries Technical Paper 402. 2000 1-53
191. Saurabh S, Rajesh K, Singh B, Mohanta KN. Nodavirus infection of giant freshwater prawn, *Macrobrachium rosenbergii* (de Man). *Aqua Int* 2005; **12(10)** : 26-27.
192. Saurabh S, Singh B, Choudhary AK. White tail disease threatens scampi industry. The Hindu, National Newspaper. 2005. 2 June, Science and Technology/Agriculture
193. Sahoo PK. White tail disease and appendage deformity syndrome-recent disease entities of economic importance of giant freshwater prawn. *Indian Farming* 2006; **56(2)** : 17-18
194. Kumar V, Saurabh S, Sahu NP, Pal AK. β -Glucan, a feed additive to manage aquatic animal health. *Aqua Feeds: Formulation and Beyond* 2005; **2(3)** : 9-11
195. Arthur JR. Fish and Shellfish Quarantine The reality for Asia-Pacific. In: Subasinghe RP, Arthur JR, Shariff M, editors. *Health management in Asian aquaculture Proceeding of the regional expert consultation on aquaculture health management in Asia and Pacific*. Rome. FAO Fisheries Technical Paper No. 360, FAO, 1996. 11-28

Impact of trawling on resource partitioning among certain demersal fishes of the Kerala coast

ASHA NAIR M* and C.M. ARAVINDAN

Department of Aquatic Biology and Fisheries, University of Kerala, Kariavattom Campus, Thiruvananthapuram, Kerala-696581

**Department of Zoology, V.T.M.N.S.S. College, Dhanuvachapuram, Thiruvananthapuram, Kerala, 695503.*

Received October 26, 2006, Revised March 29, 2007, Accepted May 9, 2007

Abstract

Bottom trawling is an important fishing method for capturing prawn and other bottom dwelling fishes which has also resulted in worldwide concern on its adverse impact on the marine fauna in general and bottom fauna in particular. There are differing opinions regarding the indirect impact of trawling on benthic fauna. According to some studies, trawling may also result in enhanced growth rate of fishes by increased availability of food. The present study is undertaken to investigate the change in resource partitioning among three demersal fishes due to trawling viz. *Nemipterus japonicus* (Bloch), *Priacanthus hamrur* (Forsskal) and *Nibea maculata* (Bloch and Schneider). Quantitative analysis of stomach contents, based on several statistical indices including diet overlap, diet breadth and index of relative importance indicate the change in dietary patterns of the fishes. Results of the study revealed interesting diet sharing patterns among the selected demersal fishes at trawled and non-trawled sites.

Key words : resource partitioning, dietary patterns, demersal fishes, trawling.

Introduction

Bottom trawling is an important fishing method for capturing prawn and other bottom living fishes. This method has resulted in worldwide concern regarding its adverse impact on the marine fauna in general and bottom fauna in particular. The impacts due to trawling have been noticed to be both physical and biological. The otter trawl, which alone is in operation in Kerala, has been reported to cause scraping, penetration and pressure of gear parts on the sediment, creating sediment suspension. These physical impacts result in habitat disturbance, mortality of bottom fauna, and exposure of bottom animals to predators and large-scale destruction of non-target species. There had been concerns regarding

सारांश

खेचू जाल से मछली फसाना मछली पकड़ने का एक प्रमुख ढंग है। इस तरीके से झींगा और दूसरी समुद्रतल में रहने वाली मछलियों को पकड़ा जाता है। पूरे विश्व में यह एक चिंता का विषय है क्योंकि इस ढंग से मछली पकड़ने पर लगभग पूरे, विशेषकर समुद्रतल में रहने वाले, जीवों पर प्रतिकूल प्रभाव पड़ता है। इस सदर्भ में वैज्ञानिकों के भिन्न-भिन्न मत हैं। कुछ के अनुसार इस व्यवस्था से भोजन की उपलब्धता बढ़ जाती है, जिससे मछलियों में बढ़ी हुई वृद्धि दर देखी जा सकती है। प्रस्तुत अध्ययन में इसका प्रभाव जिन तीन प्रजातियों की मछलियों में देखा गया वे हैं, नेमिप्टेरस जैपोनिकस (ब्लॉच), प्रियाकैन्थस हैमरक (फोर्सकल) तथा नीबिया मैक्युलाटा (ब्लॉच तथा शेनेडर)। इन मछलियों के अमाशय में मौजूद साख्यकीय अनुसूची पर आधारित आहार अतिव्याप्त तथा आहार विस्तार के मात्रात्मक विश्लेषण से इनके भोजन पद्धति में परिवर्तन इंगित होते हैं। इस अध्ययन का परिणाम, चयनित अधोजलीय मछलियों में दोनों स्थानों से, जहाँ महाजाल का प्रयोग हो रहा है या नहीं, रुचिकर, आहार सहभागी प्रतिरूप प्रकट करता है।

सांकेतिक शब्द : युक्ति विभाजन, आहार पद्धति, डिमर्सल मछलियाँ, जाल द्वारा मछली पकड़ना।

the decline in fisheries due to obstruction of spawning and large scale destruction of spawners of fin fishes and prawns during monsoon season. This has led to the implementation of trawling ban during monsoon throughout Kerala coast. Several studies in Europe have revealed that long term trawling may affect and shift community composition of macro benthos¹. Most of these studies were indirect. Only a few direct observations have been reported.

However, there are differing opinions regarding the indirect impact of trawling on benthic fauna. Trawling² may also result in enhanced growth rate of fishes by increased availability of food. Benthic predators may be benefited by the shift in species and size composition of the bottom fauna.

The present study was undertaken to investigate the change in resource partitioning among three demersal fishes due to trawling.

Material and Methods

The fishes selected for the present study were *Priacanthus hamrur* (Forsskal), *Nemipterus japonicus* (Bloch) and *Nibea maculata* (Bloch and Schneider). These fishes live near the bottom upto 20-60m of depth and are present throughout the western Indian Ocean. They feed on a wide range of bottom living animals including worms, crustaceans, cephalopods and fishes. The diet changes little with size, as small fishes prefer small crustaceans (copepods and ostracods).

Two study sites were selected, one a well known trawling ground (Neendakara) and another an important fish-landing centre (Vizhinjam) which is basically a non-trawling site. Vizhinjam, is a small village situated about 16 km south of Trivandrum (Long 76° 59' 15" E, Lat. 8° 22' 30" N) is an important fish landing centre among the twenty seven fish landing villages in the fishery zone. The Vizhinjam coast is characterized by rocky inshore realms coupled with offshore areas of hard bottom with shallow ridge-like features which rise 2-5 m from the ground level, with an irregular profile. Of the four principal fishing methods used, fishing by trawls is not practised at Vizhinjam due to the rocky nature of the sea bottom. The methods practised in this coast are the use of seines, driftnets and hook and lines. Among the various gears employed at Vizhinjam, the most important one that contributes to the bulk in perch landings is the hook and line method in the mechanised sector. Most of the sciaenid catch obtained by konchu vala and boat seine is *Nibea maculata*.

The fishing village of Neendakara is situated 9 km north of Quilon (8° 53' - 9° 02' N and 76° 31' - 76° 41' E) on the banks of Ashtamudi channel which connects Ashtamudi lake with the sea. The sea off Neendakara is a potential trawling ground for prawns and other ground fishes. This area is reported generally to be sandy and muddy.

As seen from the fishing operations using otter trawl net and gill net, the CPUE for trawling is always higher than that for gill netting⁴.

Monthly samples were collected from the trawl catches of Neendakara fishing harbour and from the

fish landing centre at Vizhinjam for a period of one year from August 2001 to July 2002.

The stomach contents were analyzed by employing points (volumetric) method. Since volumetric method alone is inadequate to give a correct picture of the dietary importance⁵, both occurrence and volume have been taken into consideration.

The percentage of each category of stomach to the total number of stomachs examined in a month was calculated and these values stand for feeding intensity. With a view to assess the feeding intensity, 'feeding index' was worked out according to the procedure of Kow⁶.

Volume of each food item and its percentage in the total volume of all stomach contents were calculated monthwise by points method. The percentage occurrence of different food items was determined monthwise from the total number of occurrence of all items. To evaluate the importance of each food item, the 'index of preponderance' proposed by Natarajan and Jhingran⁵ was followed. This method simultaneously takes into account both volumetric as well as occurrence methods in respect of individual food elements based on their relative importance. Bhatnagar and Karamchandani⁷ adopted and modified the above method for grading. The formula for calculating the index of preponderance is $V+O/2$ where 'V' is the percentage composition and 'O' is the percentage occurrence.

The gastrosomatic index (Ga.SI) was calculated using the formula,

$$\text{Ga.SI} = \frac{\text{Weight of the gut}}{\text{Weight of the fish}} \times 100.$$

Coefficient of condition or condition factor was determined by the Fulton's formula.

The data from the dietary analysis were used to calculate diet breadths, using the niche width index (B) determined by Levins⁸.

$$B = [\sum P_i^2]^{-1}$$

where P_i is the proportion of the i -th item in the diet. This index was used to compare 'specialist' tendencies between species, with low values of the index indicating 'specialists' and high values, 'generalists'. The values of B increase from 1 as the diet becomes

broader. Diet overlap was estimated by comparing the proportion of diet for pair wise species (Schoener⁹).

$$\alpha = 1 - 0.5 \left(\sum_{i=1}^n |P_{xi} - P_{yi}| \right)$$

where n is the number of food categories, P_{xi} = proportion of food category i in the diet of species x , and P_{yi} = proportion of food category i in the diet of species y .

Dietary similarity of the same species of fish between Vizhinjam and Neendakara is computed using the following index :

$$S = \frac{\sum_{i=1}^S \min(P_{ix}^V, P_{ix}^N)}{\sum_{i=1}^S \max(P_{ix}^V, P_{ix}^N)}$$

where P_{ix}^V and P_{ix}^N are the proportion of the i -th item of species x at Vizhinjam and Neendakara respectively, and S is the total number of food categories. This formula is a modified form of Czekanowski's Quantitative index. Here the percentage composition of the i -th food category has been used, and in the index, the minimum value is normalized with respect to the maximum value. The index takes the value '0' when the diets contain no items in common between the two stations, and it takes the value of 1 when the diet contains identical items. So this index gives an indication of similarity in the dietary components of the same fish at two locations.

Asymmetrical diet overlaps were calculated using MacArthur and Levins¹⁰ and Wallace¹¹ formulae:

Levins and Horn's formulae are:

$$\phi_{ij} = \frac{\sum_{i=1}^S p_{ik} p_{ij}}{\sum_{i=1}^S p_{ij}^2}$$

$$\phi_{ijk} = \frac{2 \cdot \sum_{i=1}^S p_{ik} p_{ij}}{\sum_{i=1}^S p_{ij}^2 + \sum_{i=1}^S p_{ik}^2}$$

where P_{ik} is the percentage composition of the food item i consumed by the fish k and p_{ij} is the % composition of the food item i consumed by the fish j at the same site. The pair (j, k) is taken as any one of the pairs of fishes. These measures reflect the differences in the magnitude of overlap between two fish populations due to unequal niche breadths. These

indices are computed for a pair of fish at the same site. This asymmetrical measure can be viewed as a reflection of the unequal consumption of food items available at the same site by two competing fishes.

The quantitative importance of each component in the diet was determined by the index of relative importance (IRI)^{3,12,13} which is defined as $IRI = \%F (\%N + \%V)$ where $\%F$ = frequency of occurrence of the food item, $\%N$ = numerical percentage of a food item in the stomachs, and $\%V$ = percentage by volume of the food item in the stomachs. In the present study, $\%F$ was taken as the % occurrence of a food item, and $\%N$ as the average composition of a food item.

Total content of a particular food item is taken as weighted sum of the food item and $\%V$ as the percentage composition.

The % IRI is expressed as $\%IRI = (IRI / \sum IRI) \times 100$. This modified formula of IRI is based on the reason that it is realistic to base assessment of dietary importance upon unrelated methods³, and that the indices combining values from different sources are more representative¹⁴. Data on non-target benthic fauna collected from the trawl grounds were also analyzed.

Results and Discussion

All the three species of fishes selected for the present study constitute important components of the demersal fishery resource of India in general and that of west coast in particular¹⁵⁻²³. Their importance in the fish catch from both Vizhinjam and Neendakara has also been reported by fishery biologists^{15,16,20}. At Vizhinjam, they are caught by non-mechanised and mechanised boats using conventional fishing gears like konchuvalla, boat seine, hooks and line, drift net etc., while at Neendakara the main fishing gear used is trawl net. On many occasions they constitute by-catch of prawns from trawling boats of different sizes.

Of the three species selected for the present study, the biological aspects, especially food and feeding habits of *Nemipterus japonicus* have been well documented²⁴⁻³². However, only limited information exists regarding *Priacanthus hamrur* and *Nibea maculata*^{23,29}. Observations made earlier by many authors have revealed that all the three fishes are predatory carnivores and bottom feeders^{22,23,27,31}. Food of these fishes, consists of crustaceans like squilla, prawns, mysids and other

Table 1 - Table showing values of Diet Overlap among the three species of fishes collected during the three seasons

	Pre-monsoon			Monsoon			Post-monsoon			Yearly		
Vizhinjam	Nm	Nj	Ph	Nm	Nj	Ph	Nm	Nj	Ph	Nm	Nj	Ph
<i>Nibea maculata</i> (Nm)	-	0.478	0.522	-	0.723	0.593	-	0.542	0.413	-	0.591	0.558
<i>Nemipterus japonicus</i> (Nj)	-	-	0.564	-	-	0.68	-	-	0.488	-	-	0.652
<i>Priacanthus hamrur</i> (Ph)	-	-	-	-	-	-	-	-	-	-	-	-
Neendakara												
<i>Nibea maculata</i> (Nm)	-	0.615	0.667	-	0.659	0.562	-	0.834	0.704	-	0.724	0.688
<i>Nemipterus japonicus</i> (Nj)	-	-	0.639	-	-	0.721	-	-	0.738	-	-	0.756
<i>Priacanthus hamrur</i> (Ph)	-	-	-	-	-	-	-	-	-	-	-	-

groups like squids, teleostean fishes, lamellibranch molluscs, annelids etc. with a preference for prawns and crabs.

The data pertaining to monthly variations in the feeding intensity, feeding index and the gastrosomatic index values were studied. These values also showed the similar trends in the feeding habits. The study indicated a positive relationship between feeding intensity and gastrosomatic index. The GaSI values indicate that the feeding conditions at Neendakara are better than that at Vizhinjam, except in the case of *Nibea maculata* during monsoon season. The condition coefficient was generally high in all the three fishes during the monsoon season. The condition indices are generally comparable for the three fishes at the two sites.

It may be noted that the diet overlap (Schoener index) that exist within the three fish species from Neendakara is appreciably larger than that observed at Vizhinjam (Table 1). The Schoener index of diet overlap assumes no competition (interaction) among the participating fish species for various food items. Resource availability is assumed to be unlimited, and this can generally be taken to be true in the case of the two

Table 2 - Table showing dietary similarity indices of the three fishes from the two sites

Fish species	Pre-monsoon	Monsoon	Post-monsoon	Yearly
<i>Nibea maculata</i>	0.37	0.40	0.55	0.52
<i>Nemipterus japonicus</i>	0.57	0.54	0.39	0.60
<i>Priacanthus hamrur</i>	0.57	0.57	0.47	0.63

sites considered here. Schoener index is treated as adequate to explain overlap values in the absence of resource availability data¹¹. This index reflects sharing of major food categories among the fish groups. Comparing the values in the table, it may be observed that the overlap values at Neendakara are generally higher than the corresponding values at Vizhinjam. At Vizhinjam, comparatively moderate overlap exists between the three fish groups during the monsoon season (60% to 80%) whereas at Neendakara, the overlap is more pronounced during the post-monsoon season (more than 70%). This indicates that the resources available are shared by the three species of fishes at Neendakara, in a wider scale than that at Vizhinjam. Dietary similarity of the three fishes from the two study sites is presented in Table 2. The values of similarity index show that the dietary composition of the fishes at the two sites differs significantly.

The diet overlap indices given by Levins and Horn are calculated with the assumption that each category of fish utilizes certain fraction of the food items available to another category of fish so as to effect competition for food among the fishes living in the same environment. Comparison of the values of Horns index for the two study sites (Table 3) shows that the three fish species at Neendakara share the resources among themselves on a wider scale than the fishes at Vizhinjam. Comparison of the diet breadth data of the three fishes at Vizhinjam and Neendakara (Table 4) does not show any clear trend. The Index of Relative Importance (IRI) of each food component in the diet was calculated and presented in Tables 5, 6 and 7. Seasonal changes in the preferred food items of the three fish species were observed from the data.

Table 3 - Asymmetrical diet overlaps of the three fishes at Vizhinjam and Neendakara. (Based on Levins and Horn formula)

Index	Vizhinjam				Neendakara			
	Pre-monsoon	Monsoon	Post-monsoon	Yearly	Pre-monsoon	Monsoon	Post-monsoon	Yearly
Levins _{NMG1}	0.53	0.81	0.65	0.63	0.57	0.71	0.94	0.83
Levins _{NJg1}	0.97	0.92	0.83	0.89	0.74	1.11	1.01	0.93
Horn _{NM,NJg1}	0.69	0.86	0.73	0.74	0.64	0.87	0.97	0.87
Levins _{NJg2}	0.67	0.94	0.52	0.78	0.77	0.79	0.84	0.83
Levins _{PRg2}	0.89	0.80	0.66	0.82	0.96	1.07	1.00	1.08
Horn _{NJ,PRg2}	0.76	0.86	0.58	0.80	0.86	0.91	0.92	0.93
Levins _{NMG3}	1.03	0.79	0.74	0.80	1.00	1.25	1.00	1.07
Levins _{PRg3}	0.42	0.82	0.45	0.53	0.61	0.51	0.79	0.75
Horn _{NM,PRg3}	0.60	0.80	0.56	0.63	0.77	0.80	0.88	0.87

Table 4 - Table showing values of Diet Breadth of the three species of fishes collected during the three seasons

Vizhinjam	Pre-monsoon	Monsoon	Post-monsoon	Yearly
<i>Nibea maculata</i>	3.66	5.29	3.32	4.37
<i>Nemipterus japonicus</i>	6.64	6.02	4.25	6.24
<i>Priacanthus hamrur</i>	8.84	5.11	5.44	6.62
Neendakara				
<i>Nibea maculata</i>	4.15	3.12	5.00	4.90
<i>Nemipterus japonicus</i>	5.42	4.88	5.39	5.48
<i>Priacanthus hamrur</i>	6.74	6.62	6.42	7.15

Results of the present study conform to the previous observations made by several authors^{4,22,23,27}. In both Vizhinjam and Neendakara all the three species feed on almost similar items of food. The higher diversity of food items indicates the diversity of food items present in the benthic habitat of both the study sites. The present results also indicate that they are selective predators preferring larger animals like fishes, prawns, squilla, squids etc. According to the optimal foraging theory, predatory fishes prefer larger prey to obtain more energy with optimum effort³³⁻³⁵. It is evident that not much variations exist in the diversity of food items consumed by these fishes and their quantity during different seasons. Similar observations on *Nemipterus japonicus* and *Priacanthus hamrur* have been made during previous studies^{22,23,31,36}.

Since feeding habits of these fishes reported from different areas of west coast and east coast of India

show similarities in the diet composition and preference, the variation in percentage composition of food items from Vizhinjam and Neendakara fishing areas found during the present study can be attributed to the difference in their abundance in these two areas which in turn may be due to the degree of fishing pressure on these animals in both places. The high intensity of trawling in Neendakara might be affecting the benthic animals due to trawling which may indirectly be influencing the food preference of these fishes. Among the three species of fishes also variations in percentage composition of food items and their rank in abundance could be noticed. While index of preponderance values were compared, the difference in the ranking of food items observed may also be due to the variations in the abundance of these bottom fauna. However, when there is intense predation, there may be more competition for specific food items within the same habitat/ environment.

Table 5 - Comparison of values of Index of Relative Importance in *Nibea maculata* collected from Vizhinjam and Neendakara

<i>Nibea maculata</i>	Vizhinjam						Neendakara					
	IRI			% IRI			IRI			% IRI		
	Post-monsoon	Pre-monsoon	Monsoon	Post-monsoon	Pre-monsoon	Monsoon	Post-monsoon	Pre-monsoon	Monsoon	Post-monsoon	Pre-monsoon	Monsoon
Fish	0.00	0.00	0.00	0.00	0.00	0.00	495.34	5101.79	0.00	2.00	14.17	0.00
Fish larvae	0.00	36.43	0.00	0.00	0.08	0.00	0.00	10.91	0.00	0.00	0.03	0.00
Fish fragmnt	1002.97	20628.32	7832.04	21.37	44.25	30.02	6006.08	6100.63	3761.73	24.31	16.95	23.29
Fish Eggs	0.00	1772.62	0.00	0.00	3.80	0.00	448.96	130.91	0.00	1.82	0.36	0.00
Harp copepods	0.00	517.13	412.42	0.00	1.11	1.58	5.42	9.69	0.00	0.02	0.03	0.00
Isopods	0.00	0.00	23.06	0.00	0.00	0.09	0.00	0.00	0.00	0.00	0.00	0.00
Amphipods	0.00	272.91	0.00	0.00	0.59	0.00	13.55	205.75	0.00	0.05	0.57	0.00
Ostracods	0.00	0.00	0.00	0.00	0.00	0.00	5.42	74.23	0.00	0.02	0.21	0.00
Mysids	0.00	709.84	723.39	0.00	1.25	2.77	6.78	37.79	0.00	0.03	0.10	0.00
Euphausiids	0.00	0.00	0.00	0.00	0.00	0.00	31.61	0.00	0.00	0.13	0.00	0.00
Prawn	136.72	18.87	3274.32	2.91	0.04	12.55	282.33	776.55	59.09	1.14	2.16	0.37
Crab-megalopa	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Crab	364.77	0.00	0.00	7.77	0.00	0.00	93.63	12.48	344.50	0.38	0.03	2.13
Squilla	0.00	0.00	0.00	0.00	0.00	0.00	40.66	0.00	118.18	0.16	0.00	0.73
Almularva	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Lucifer	0.00	69.50	551.63	0.00	0.15	2.11	0.00	0.00	0.00	0.00	0.00	0.00
Cumaceans	0.00	0.00	0.00	0.00	0.09	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Crustacean fragments	182.11	319.78	186.99	3.88	0.69	0.72	580.17	1142.05	1388.93	2.35	3.17	8.60
Sepia	0.00	0.00	0.00	0.00	0.00	0.00	18.08	0.00	0.00	0.07	0.00	0.00
Loligo	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Bivalves	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Gastropods	0.00	3.14	0.00	0.00	0.01	0.00	5.42	47.39	0.00	0.02	0.13	0.00
Nematodes	0.00	0.00	473.16	0.00	0.05	1.81	234.08	132.83	0.00	0.95	0.37	0.00
Nereis	0.00	25.46	567.56	0.00	0.05	2.18	216.81	41.19	0.00	0.88	0.11	0.00
Digested matter	3007.27	22246.83	12040.70	64.07	47.72	46.15	16225.78	22177.96	10479.48	65.66	61.60	64.88
Unidentified	0.00	0.00	11.57	0.00	0.00	0.04	0.00	0.00	0.00	0.00	0.00	0.00

Table 6 - Comparison of values Index of Relative Importance in *Nemipterus japonicus* collected from Vizhinjam and Neendakara.

<i>Nemipterus japonicus</i>	Vizhinjam						Neendakara					
	IRI			% IRI			IRI			% IRI		
	Post-monsoon	Pre-monsoon	Monsoon	Post-monsoon	Pre-monsoon	Monsoon	Post-monsoon	Pre-monsoon	Monsoon	Post-monsoon	Pre-monsoon	Monsoon
Fish	0.00	764.40	71.77	0.00	6.19	0.67	237.35	0.00	41.66	1.24	0.00	0.05
Fish larvae	0.00	0.00	0.00	0.00	0.00	0.00	0.00	7.77	0.00	0.00	0.02	0.00
Fish fragment	644.32	2018.33	779.69	3.22	16.35	7.24	2396.86	5849.71	7251.78	12.49	14.60	9.14
Fish Eggs	148.69	87.21	75.57	0.74	0.71	0.70	525.13	59.55	58.33	2.74	0.15	0.07
Harp. copepods	0.00	0.00	7018	0.00	0.00	0.07	0.00	0.00	9.59	0.00	0.00	0.01
Isopods	0.00	0.00	0.00	0.00	0.00	0.00	5.51	43.94	5.21	0.03	0.11	0.01
Amphipods	0.00	713.79	0.00	0.00	5.78	0.00	8.24	5.54	0.00	0.04	0.01	0.00
Ostracods	0.00	54.95	0.00	0.00	0.45	0.00	84.06	35.60	37.41	0.44	0.09	0.05
Mysids	0.00	19.71	123.01	0.00	0.16	1.14	0.00	31.47	1626.99	0.00	0.08	2.05
Euphausiids	0.00	0.00	0.00	0.00	0.00	0.00	26.37	0.00	0.00	0.14	0.00	0.00
Prawn	58.68	217.34	1600.43	0.29	1.76	14.86	34.94	2511.21	6654.02	0.18	6.27	8.39
Crab-megalopa	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Crab	226.54	234.75	0.00	1.13	1.90	0.00	13.19	925.94	1345.99	0.07	2.31	1.70
Squilla	1968.65	0.00	0.00	9.83	0.00	0.00	0.00	4.10	271.85	0.00	0.01	0.34
Alimular	0.00	18.94	0.00	0.00	0.15	0.00	0.00	0.00	2489.94	0.00	0.00	3.14
Lucifer	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Cumaceans	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Crustacean fragments	6198.97	2744.08	1338.34	36.95	22.23	12.43	1165.63	5685.56	2568.23	6.07	14.19	3.24
Sepia	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Loligo	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Bivalves	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Gastropods	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Nematodes	22.07	797.42	513.86	0.11	6.46	4.77	1528.51	2227.92	742.21	7.96	5.56	0.94
Nereis	0.00	0.00	41.05	0.00	0.00	0.38	578.96	102.09	0.00	3.02	0.25	0.00
Digested matter	1762.49	4675.37	6195.83	53.73	31.87	57.54	12586.17	22579.96	56263.85	65.58	56.35	70.88
Unidentified	0.00	0.00	20.49	0.00	0.00	0.19	0.00	0.00	0.00	0.00	0.00	0.00

Table 7 - Comparison of values Index of Relative Importance in *Pracanthus hamrur* collected from Vizhinjam and Neendakara.

<i>Pracanthus hamrur</i>	Vizhinjam						Neendakara					
	IRI			% IRI			IRI			% IRI		
	Post-monsoon	Pre-monsoon	Monsoon	Post-monsoon	Pre-monsoon	Monsoon	Post-monsoon	Pre-monsoon	Monsoon	Post-monsoon	Pre-monsoon	Monsoon
Fish	551.87	1102.66	317.38	0.33	0.68	0.23	676.06	4506.59	2445.64	0.78	3.25	1.79
Fish larvae	0.00	0.00	0.00	0.00	0.00	0.00	0.00	45.23	71.88	0.00	0.03	0.05
Fish fragment	1358.45	1967.95	2707.87	0.82	1.22	1.95	3639.96	8526.60	11006.69	4.18	6.14	8.07
Fish Eggs	20146.05	13612.42	2086.13	12.21	8.42	1.50	6912.26	8070.49	9985.07	7.94	5.82	7.32
Harp copepods	0.00	6198.92	83.19	0.00	3.83	0.06	161.68	90.24	26.94	0.19	0.07	0.02
Isopods	129.59	5599.86	85.45	0.08	3.46	0.06	16.64	56.15	0.00	0.02	0.04	0.00
Amphipods	102.53	1306.57	101.16	0.06	0.81	0.07	728.36	1620.41	0.00	0.84	1.17	0.00
Ostracods	6.33	835.26	129.17	0.00	0.52	0.09	232.19	1155.05	1116.89	0.27	0.83	0.82
Myids	175.56	3623.42	11029.28	0.11	2.24	7.94	23.48	4659.83	9889.05	0.03	3.36	7.25
Euphausiids	43.09	243.15	788.13	0.03	0.15	0.57	5591.00	19.01	1023.89	6.42	0.01	0.75
Prawn	63496.43	20402.55	44842.17	38.50	12.62	32.27	840.39	25440.15	8320.40	0.97	18.33	6.10
Crab-megalopa	0.00	785.41	33.48	0.00	0.49	0.02	84.33	407.41	20.48	0.10	0.29	0.00
Crab	885.49	4308.55	578.10	0.54	2.54	0.42	1130.52	165.99	577.20	1.30	0.12	0.42
Squilla	60.86	4036.25	0.00	0.04	2.50	0.00	14.97	42.29	0.00	0.02	0.03	0.00
Alimnialarva	0.00	1918.17	0.00	0.00	1.19	0.00	0.00	169.06	0.00	0.00	0.12	0.00
Lucifer	0.00	0.00	0.00	0.00	0.00	0.00	6024	1.84	0.00	0.01	0.00	0.00
Cumaceans	0.00	0.00	11.35	0.00	0.00	0.01	0.00	0.00	0.00	0.00	0.00	0.00
Crustacean fragments	24463.84	14179.70	8008.64	14.83	8.77	5.76	13213.64	10359.45	14007.87	15.18	7.46	10.27
Sepia	0.00	0.00	14.44	0.00	0.00	0.01	9.98	23.91	53.88	0.01	0.02	0.04
Loligo	0.00	0.00	0.00	0.00	0.00	0.00	4.99	0.00	0.00	0.01	0.00	0.00
Bivalves	0.00	0.00	34.75	0.00	0.00	0.03	0.00	4.30	0.00	0.00	0.00	0.00
Gastropods	107.21	62.78	40.31	0.07	0.04	0.03	19.96	2.45	23.06	0.02	0.00	0.00
Nematodes	987.99	292.3	0.00	0.60	0.18	0.00	96.18	210.16	2529.49	0.11	0.15	1.86
Nereis	3218.15	130.32	0.00	1.95	0.08	0.00	471.57	25.96	25.33	0.54	0.02	0.00
Digested matter	49119.14	81167.37	68029.01	29.78	50.20	48.96	53135.59	73168.85	75232.80	61.03	52.72	55.17
Unidentified	83.86	128.52	28.01	0.05	0.08	0.02	52.55	2.76	0.00	0.06	0.00	0.00

Results of the estimation of feeding intensity of the fishes and their comparison between the two sites also indicated a noticeable difference. This difference in feeding intensity is an indication regarding the availability or nonavailability of preferred food items in the environment³⁶. Comparison of the feeding index showed high intensity in Vizhinjam compared to Neendakara. Similarly the higher percentage of empty stomachs in samples collected from Neendakara is an indication of shortage of the most preferred food item in the environment, especially prawns. Trawling may be depleting the prawn resource of Neendakara area and hence samples of the fishes collected from this area may naturally be having shortage of that item in the environment. This is being reflected in the feeding intensity also. Results on gastrosomatic index and the condition factor of fishes support this view. Further, it is noticed that though, the three fishes share almost the same prey items; there exists variations in the similarity index among the three species and also among the different seasons.

The diet overlap values (Schoener index) help in assessing the competition among species and also in comparing different habitats without information on resource availability¹¹. The results show that the resources available are shared by the three species in a more wider scale at Neendakara than at Vizhinjam. Diet overlap values also indicate a decrease in the food resource at Neendakara than at Vizhinjam. This change in predation pattern may again be due to the high fishing pressure on the environment directly and indirectly. A decrease in the preferred items like prawns, crabs etc. might definitely be adversely affecting the food preference and predatory efficiency in that habitat. The fishes are forced to share the available food items in a wider scale at Neendakara than at Vizhinjam. While the benthic animals are sparse, diet overlap has been found to be low or in other words, different species will be exploiting different prey items³⁷. Indices of relative importance of different food items during the three seasons helped in assessing the shift in predatory pattern of the three species during the different seasons. In the post-monsoon season a different dietary pattern was observed probably due to the closed season and subsequent stabilization of the bottom fauna.

A comparison of these indices clearly indicates that Neendakara is a highly disturbed area, which affects the food resources of the species studied. In Neendakara,

these predatory fishes have to forage on more varieties of food items, probably due to the shortage of the preferred items. Nevertheless, they have to search for more prey items and also to spend more energy in foraging on more varieties of prey items. The comparatively higher diversity values observed at Neendakara and higher diet breadth supports this view.

Otter trawling and its effect on the sea bed have been studied in detail as early as in 1970³⁸. The impact on sea bed, especially trawl tracks has been reported to be 8 - 17 cm in mud and 0-5cm in sand³⁹. On muddy fine sand the impact is about 10-15 cm. Moreover, the duration of the trawl tracks will be very short due to the pattern of currents. The important effect due to the tracks is sediment re-suspension and subsequent dislocation of benthic fauna due to the turbulence created by the net. This post trawling stress may also result in habitat modification, which in turn may reduce habitat complexity, resulting in fluctuations within benthic communities^{40,41}. These fluctuations are reflected in the predatory behaviour and food partitioning of demersal fishes. Studies by Messieh *et al*⁴² have shown incidental mortality and increased susceptibility to predation of benthic animals and also alteration of chemistry and texture of the sediments. Reduction in species diversity, change in relative abundance of species etc. are also potential effects of trawling.

References

1. Groot S J de, Lindeboom HJ. Environmental impact of bottom gears on benthic fauna in relation to natural resources management and protection of the North Sea. In: NIOZ-Report 1994. p. 257.
2. De Veen JF. On changes in some biological parameters in the North Sea sole. *J Cons Int Explor Mer* 1976; **37**: 60-90.
3. Hyslop EJ. Stomach content analysis-A review of methods and their applications. *J Fish Biol* 1980; **17** : 411-429
4. Kuthalingam MDK, Livingston P, Sarma PSS. Observations on the catches of the mechanized boats at Neendakara. *Indian J Fish* 1978; **25 (1&2)** : 98-108.
5. Natarajan AV, Jhingran AG. Index of preponderance-A method for grading the food elements in the stomach analysis of fishes. *Indian J Fish* 1961; **8(1)** : 54-59.
6. Kow TA. The food and feeding relationships of the fishes of Singapore straits. U K : *Colonial office Fishery publications*, 1950; **1** . 1-35.

7. Bhatnagar GK, Karamchandani SJ Food and feeding habits of *Labeo fimbriatus* (Bloch) in River Narmada near Hoshangabad. *J Inland fish Soc India* 1970, **11** : 30-50
8. Levins R. Evolution in changing environments : Some Theoretical Explorations. Princeton · Princeton University Press, 1968.
9. Schoener TW. Non - synchronous spatial overlap of lizards in patchy habitats. *Ecology* 1970; **51** : 408-418
10. MacArthur RH, Levins R The limiting similarity, convergence and divergence of coexisting species *Am Nat* 1967; **101** : 377-385.
11. Wallace RK An assessment of diet-overlap indexes. *Trans Amer Fish Soc* 1981, **110** : 72-76.
12. Pinkas L, Oliphant MS, Iverson ILK. Food habits of albacore, blue fin tuna and bonito in California waters. *Calif Dep Fish Gamefish Bull* 1971; **152** : 1-105
13. Carrasson M, Matallanas J. Feeding ecology of the Mediterranean spiderfish, *Bathypterois mediterraneus* on the western Mediterranean slope. *Fish Bull* 2001; **99** : 266-274
14. Windell JT Food analysis and rate of digestion. In Ricker WE, editor. Methods for assessment of fish production in freshwater International Biological Programme Handbook 3, Oxford · Blackwell Scientific Publications, 1971 197-203
15. Nair SG. A preliminary account of the fisheries of Vizhinjam. *Indian J Fish* 1958; **5** : 32-54
16. Radhakrishnan N. Demersal fisheries of Vizhinjam. *Indian Fish*, 1974; **21** : 29-39.
17. Sastry AY, Chandrasekhar M. The small commercial trawl fisheries off Vishakhapatnam during 1982-83 and 1983-84 *J Mar Biol Ass India* 1986; **28 (1&2)** : 74-83
18. Naik SK. On "Bulls Eye"- the deep sea resources. *Seafood Export J* 1990; **32** : 16-18
19. Samuel M Biology, age, growth and population dynamics of thread fin bream *Nemipterus japonicus* *J Mar Biol Ass India* 1990, **32 (1&2)** : 66-76.
20. Thomas PA, Lazarus S, Vincent SG, Madan Mohan, Omana T A. Perch fishery at Vizhinjam. *Bull Cent Mar Fish Res Inst* 1994; **47** : 36-89
21. Chakraborty SK. Fishery, age, growth and stock assessment of *Priacanthus hamrur* (Forsskal) from Bombay waters. *Bull Cent Mar Fish Res Inst* 1994, **47** : 121-127.
22. Premalatha P. On the fishery and biology of *Priacanthus hamrur* (Forsskal) along the south west coast of India. *Indian J Fish* 1997; **44 (3)** : 265-270.
23. Sivakami S, Raje SG, Ferozkhan M, Shobha JK, Vivekanandan E, Rajkumar U. Fishery and biology of *Priacanthus hamrur* (Forsskal) along the Indian coast *Indian J Fish* 2001; **48 (3)** : 277-289.
24. Rao KS Food and feeding habits of fishes from trawler catches in the Bay of Bengal with observations on diurnal variations in the nature of the feed *Indian J Fish* 1967, **11 (1) A** : 277-314.
25. Murthy VS. Further studies on the growth and yield per recruit of *Nemipterus japonicus* (Bloch) from the trawling ground off Kakinada. *Indian J Fish* 1987, **34(3)** : 265-276
26. Eggleston D. Patterns of Biology in the Nemipteridae *J Mar Biol Ass India* 1972; **14(1)** : 357-364
27. Vinci GK. Thread fin bream (*Nemipterus*) resources along the Kerala coast with notes on biology of *Nemipterus japonicus* *Indian J Fish* 1983; **29** : 37-49
28. Mohan M Velayudhan AK. A few observations on the taxonomy and biology of *Nemipterus delagoae* (Smith) from Vizhinjam. *Indian J Fish* 1984; **31(1)** : 113-121.
29. Apparao T On some aspects of biology of *Priacanthus macracanthus* (Cuv.) *J Mar Biol Ass India* 1984, **31 (1 and 2)** : 103-109
30. Vivekanandan E Distribution pattern of threadfin breams along north Tamil Nadu and Andhra coasts. *Indian J Fish* 1990, **37** : 269-280.
31. Vivekanandan E. Predatory diversity of two demersal finfish species in the trawling grounds off Veraval. *Indian J Fish* 2001; **48 (2)** : 133-143.
32. Vivekanandan E Production efficiency of two demersal finfishes in the trawling grounds off Veraval *Indian J Fish* 2001; **48 (2)** : 123-132
33. MacArthur RH, Pianka ER On optimal use of patchy environment. *Am Nat* 1966; **100** : 603-609
34. Kerr SR. Prey availability, metaphoresis and size structure of lake trout stocks *Invest Pesq* 1979; **43** : 187-198
35. Krebs JR. Optimal foraging · decision rules for predators. In. Krebs JR, Davies NB. editors Behavioural ecology. Oxford · Blackwell Sci Pub, 1978; 23-63.
36. Gopal C, Vivekanandan E Threadfin bream fishery and biology for *Nemipterus japonicus* off Veraval. *Indian J Fish* 1991; **38** : 97-102.
37. Carter CG, Grove DJ, Carter DM. Trophic resource partitioning between two coexisting flatfish species off the north coast of Anglesey, Northwales. *Neth J Sea Res* 1991, **27 (3/4)** : 325-335.
38. Bridger JP. Some effects of the passage of a trawl over the sea bed. *ICES CM* 1970, **(B)** : p. 10.

- 39 Caddy JF. Under water observations on tracks of dredges and trawls and some effects of dredging on scallop ground *J Fisheries Res Board Canada* 1973; **30** : 173-180
- 40 Krost P, Bernhard M, Werner F, Hukriede W. Otter trawl tracks in Kiel Bay (Western Baltic) mapped by side-scan-sonar *Meeresforschung* 1990; **32** : 344-353
- 41 Linnane A, Ball B, Munday BW, Bob van Marlen, Magda Bergman, Fonteyne R. A review of potential techniques to reduce the environmental impact of demersal trawls *Irish Fisheries Investigations* (New Series) 2000; **7** : 1-39.
- 42 Messieh SN, Rowell TW, Peer DL, Cranford PJ. The effects of trawling, dredging and ocean dumping on the eastern Canadian continental shelf sea bed. *Continental Shelf Res* 1991; **11** : 1237-1263.

Detection of *Vibrio* proteins from diseased *Penaeus monodon* (Fab.) by ELISA and Western blotting

*N. CHANDRAKALA¹, G. SAMPATH KUMAR¹, P. REBEKKA, G. KIRUBA JASMINE, M. PRABAKARAN and M. AYYAVOO²

* Dept. of Zoology K.N. Govt. Arts College (W) Autonomous, Thanjavur - 613007

1. PG & Research Department of Microbiology, Ponnaiyah Ramajayam College Thanjavur 614904, Tamilnadu, India.

2. PG & Research Department of Zoology, A.V.V.M. Sri Pushpam College (Autonomous), Poondi 613503, Thanjavur, Tamilnadu, India.

*e-mail : nc_kala@hotmail.com

Received April 13, 2005, Revised April 12, 2006, Re-revised May 27, 2007 Accepted August 17, 2007

Abstract

Vibrio species were isolated from black spot diseased *Penaeus monodon*. Proteins were isolated from bacteria and subjected to Enzyme Linked Immuno Sorbent Assay (ELISA). The pathogenic protein was blotted by Western blotting. SDS-PAGE analysis revealed the presence of 29 kDa protein unique to pathogenic condition. The antigens were detected by ELISA.

Key words: shrimp, ELISA, SDS-PAGE, blotting.

Introduction

The prawn/shrimp industry is facing serious problem with several types of diseases caused by bacteria, fungi and viruses. Bacteria of the genus *Vibrio* are ubiquitous in the marine and estuarine aquatic ecosystem in which shrimp occur naturally and are farmed¹. All life stages of penaeid shrimp may be affected². Antigen can be detected or measured by Enzyme Linked Immuno Sorbent Assay (ELISA)³ in which the antibody is immobilized. A sample containing antigen is added and allowed to react. A second enzyme linked antibody, specific for different epitope on the antigen is added. After adding the substrate the colored reaction product is measured. The protein mixture is electrophoresed, the protein bands are transferred to a nitrocellulose membrane with enzyme linked polyclonal or monoclonal antibody specific for the protein of interest⁴. Hence

सारांश

ब्लैक स्पॉट रोग से पीड़ित *पीनियस मॉनोडॉन* के शरीर से *वाइब्रियो* स्पीसीज को प्रथक्करित किया गया। उक्त बैक्टीरिया से प्रथक्करित प्रोटीन का एलीसा तकनीक द्वारा अध्ययन किया गया। इसके हानिकारक प्रोटीन को वेस्टर्न ब्लॉटिंग द्वारा सशक्त किया गया। एसडीएस-पेज प्रक्रिया द्वारा इसमें ऐसे विशिष्ट 29 kDa प्रोटीन मिले जो हानिकारक व्यवस्था से संबद्ध थे। एलीसा तकनीक द्वारा इसके एन्टीजन पहचाने गये।

सांकेतिक शब्द : झींगा, एलीसा, एसडीएस-पेज, ब्लॉटिंग

an attempt has been made to detect the *Vibrio* protein by ELISA and Western blotting.

Material and Methods

The diseased prawn showing black spots were collected from culture ponds of Mallipattinam, Tamil Nadu, India. Swab was plated on Thiosulphate Citrate Bile salt Sucrose (HiMedia, Mumbai) (TCBS>pH8.5 ± 0.2) agar, incubated for 24hrs at 37°C. Colour and morphology of the colony were recorded and subjected to various biochemical tests.⁵ By comparing the results of biochemical tests with Bergey's Manual of Determinative Bacteriology⁶ and a set of keys for biochemical identification of environmental *Vibrio* sp⁷, the isolates were characterized. The total protein of diseased and control *P.monodon* and isolates of *Vibrio* sp were estimated by Biuret method⁸. The protein were

Table 1 - Biochemical Characteristics of *Vibrio* spp.

S. No		Characteristics		
		<i>V. parahaemolyticus</i>	<i>V. orientalis</i>	<i>V. mediterranei</i>
1.	Colour of the colony on TCBS Agar	Green	Yellow	Yellow
2.	Colony morphology	Smooth	Granular	Mucus
3.	Gram's staining	-ve Rod	-ve Rod	-ve Rod
4.	Size of the colony (mm)	3	3	3
5.	Cell Size (Length - mm)	0.8	0.8	2.56
6.	Diameter	0.3	0.3	6.1
7.	Motility	+	+	+
8.	Indole	-1	4-	4-
9.	Methyl Red	-	-	+
10.	Voges Proskauer	-	-	-
11.	Citrate	+	+	+
12.	Catalase	+	+	+
13.	Oxidase	+	+	+
14.	Urea hydrolysis	+	+	+
15.	Dextrose	A	A	A
16.	Sucrose	A	A	A
17.	Lactose	-	-	A
18.	Maltose	AG	A	A
19.	Mannitol	A	A	+
20.	Arabinose	+	A	A
21.	Growth in 0% NaCl	+	+	+
22.	Growth in 1%NaCl	-	+	-
23.	Growth in 6% NaCl	+	+	+
24.	Growth in 8% NaCl	-	-	+
25.	Growth at 4°C	-	-	-
26.	Growth at 30°C	+	+	+
27.	Growth at 40°C	-	-	-
28.	Growth in pH4	+	+	-
29.	Growth in pH9	+	+	+
30.	Gelatin hydrolysis	-	-	+
31.	Starch hydrolysis	-	+	+
32.	Ciprofloaxin sensitivity	+	+	+
33.	Luminescence	-	-	-

Note : A - Acid, AG - Acid and Gas, + - Presence of growth, - - Absence of growth

Table 2 - Total Protein of Carapace and *Vibrio* Isolates.

Sample/Isolate	Replicates	%	X \pm SD
Control Sample	1	12.4	12.566 \pm 0.152
	2	12.6	
	3	12.7	
Infected Tissue	1	12.09	12.10 \pm 0.01
	2	12.11	
	3	12.10	
<i>V. cholerae</i>	1	10.7	10.466 \pm 0.251
	2	10.5	
	3	10.2	
<i>V. diazotrophicus</i>	1	11.0	11.166 \pm 0.153
	2	11.2	
	3	11.3	
<i>V. harveyi</i>	1	12.1	12.3 \pm 0.264
	2	12.2	
	3	12.6	

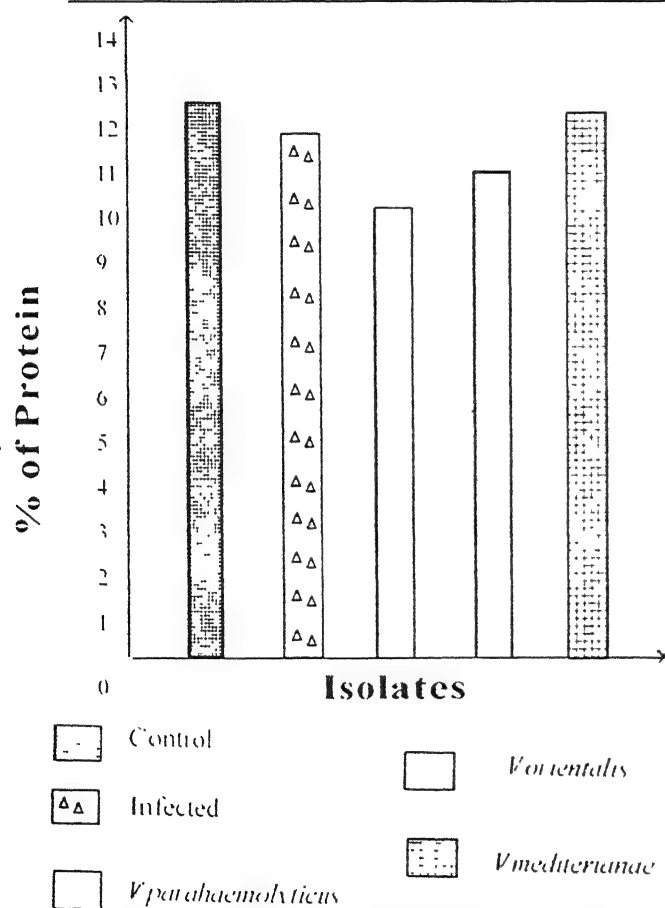
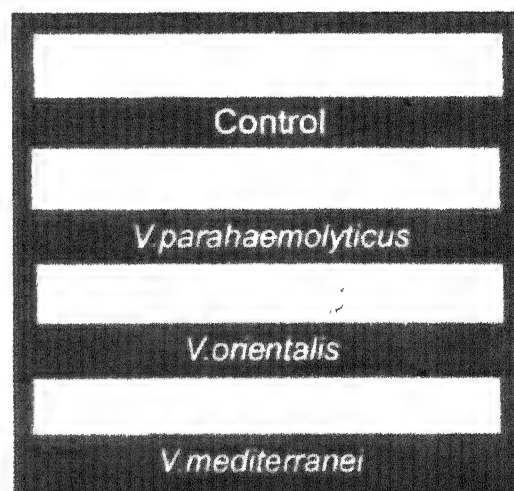
Fig. 1 - Total protein of Tergum and *Vibrio* isolates

Fig. 2-A - Appearance of Blue spot

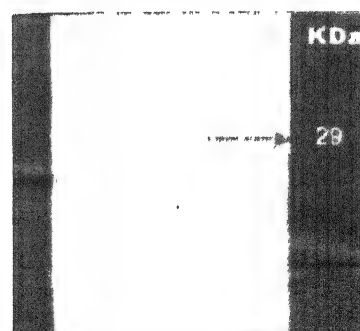


Fig 2-B - Blotting of 29 Kda protein band on Nc membrane

separated by Sodium Dodecyl Sulphate PolyAcrylamide Gel Electrophoresis (KT#30, GENEI, BANGALORE). The gel was removed and stained with Eze blue for 1 hr. After destaining the protein band was subjected to electroblotting⁹ over nitrocellulose membrane (NC). The NC membrane was placed in blocking buffer (O N at 4°C) V immersed in primary antibody and transferred to labeled secondary antibody followed by the addition of substrate (10 mins). The blot was removed, washed and air dried. DOT ELISA was performed¹⁰ (GENEI BANGALORE).

Results and Discussion

The diseased prawn showed black spot on the tergum, on TCBS agar green and yellow colour colonies were observed. The results of biochemical tests revealed the presence of *V. parahaemolyticus*, *V. orientalis* and *V. mediterranei* (Table 1). The total protein showed significant variation ($P < 0.05$; Table 2 and Fig 1). The DOT-ELISA revealed the presence of blue spot (Fig 2A). SDS-PAGE analysis revealed the presence of 29 kDa in *V. orientalis* and *V. mediterranei* and 38 kDa protein in *V. parahaemolyticus*. Western blot analysis showed the transfer of 29 kDa protein on NC membrane and was intensively stained (Fig 2B). Black spot was observed on the tergum¹¹. The bacterial profile of water from the farms on the West Coast revealed that *V. parahaemolyticus* ranged from 2.8% to 26% and were negative for haemolytic activity on Wagatsuma agar¹². The serotype specific polyclonal antibodies to *V. cholerae* 0139 were usually prepared by repeated immunization of rabbits over an extended period of time by using *V. cholerae* whole cells as the immunogen¹³. Tamil selvan¹⁴ observed similar results in the presence of blue spots using *V. cholerae* whole cells as the immunogen.

SDS-PAGE and Western blot analysis carried out in the present study revealed the presence of protein band corresponding to 29 kDa. Manikandan¹⁵ observed 29 kDa protein unique to the pathogenic strains of *Leptospira*.

References

1. Ruangpan L, Kitao T. *Vibrio* bacteria isolated from black tiger Shrimp *P. monodon* (Fab.) *J Fish Dis* 1991; 4 : 383- 388.
2. Lighter D V. Crustacean Aquaculture. In: Mcvey J P, editor. Disease of cultured penaeid shrimp. Boca Raton: CRC Press 1993. 393 - 486.
3. Goldsby AR, Kindt JT, Osborne AB. Immunology. IV edition New York : W.H. Freeman and company, 1999.
4. Aneja KR Biochemical activities of microorganism Experiments in Microbiology, Plant pathology. Tissue culture and Mushroom production technology. III edition, New Delhi . New Age International Publishers, 1994.
5. Holt JG, Krieg NR, Sneath PHA, Staley JT, Williams ST. Bergey's Manual of Determinative Bacteriology, IX edition New york, 1994.
6. Alsina M, Blanch AR A set of keys for biochemical Identification of environmental *Vibrio* species. *J Applied Bacteriol* 1994; 61 : 431 - 437.
7. Gornall G, Bardawill CJ, David MM. Determination of total serum protein by means of biuret reaction *J Biochem* 1949, 177 : 751-766.
8. Gooderham K. Protein blotting techniques in Molecular Biology. In: Walker JM, Gasshra W, editors. London : Croom Helm Publishers, 1983.
9. Voller A, Bartlett A, Bodwell DE. Enzyme immuno assays with special reference to ELISA Technique. *J Clinical Pathology* 1978; 31 : 507-520.
10. Anderson IG, Shamsudin MM, Shariff M, Nash G. Bacterial septicemia in juvenile tiger shrimp *Penaeus monodon* cultured in Malaysian brackish water ponds. *Asian Fish Sci* 1998; 2(1) . 93-108.
11. Otta SK, Karunasagar I. Bacterial flora associated with shrimp culture ponds growing *Penaeus monodon* in India. *J Aqua Tropics* 1999; 14 (4) : 309-318.
12. Song G, Kun Z, Dasheng X, Rukuan Z, Xiu Fan L, Gao S, Zhao K, Xu DS, Zhang RK, Liu XF. Development of an indirect ELISA for detecting antibodies against outer membrane proteins of *E coli* isolates from chickens. *Cli J Vet Sci* 1999; 19(4) : 336-338.
13. Tamilselvan S. Standardization of Dipstick ELISA for *V. cholerae*. M.Phil dissertation. Bharathidasan University, Trichirapalli, 2003.
14. Manikandan P. SDS - PAGE and Western blot analysis of Leptospiral proteins. M.Phil dissertation. Bharathidasan University, Tiruchirappalli, 2003.

Differential Giemsa staining patterns in chromosomes of three Iranian species of *Fritillaria* spp. group

GHOLAMREZA BAKHSHI KHANIKI

Payame Noor University, P.O. Box 19395-4697, Tehran, Iran

e-mail : Bakhshi@pnu.ac.ir

Abstract

On the basis of C-band patterns and other karyologic features three species are described for the Iranian *Fritillaria* spp. group, *F. caucasica* (Adams), *F. uva-vulpis* (Rix) and *F. assyriaca* (Baker). All the three species have a similar basic karyotype ($n=12$), consisting of large symmetric (m, sm) and smaller asymmetric (st, t) chromosomes, but C-bands differ between them. The bands are located at centromeric, intercalary, and telomeric positions and adjacent to secondary constrictions. *F. caucasica* and *F. assyriaca* differ from others in the possession of a submetacentric chromosome pair. Further aspects of constitutive heterochromatin, heteromorphism, equilocal C-band distribution, C-banding evolution, and the role of C-banding in taxonomy are discussed.

Key words *Fritillaria* species group, C-banding, karyotypes, heterochromatin

Introduction

As part of a larger research programme on the systematics and evolution of liliaceous plant groups of South West Asia and the Mediterranean, the author previously studied the Giemsa C-banded karyotypes in Iranian species of *Rhinopetalum* and *Fritillaria* subgen. *Petilium* and *Theresia* (Bakhshi Khaniki 1998)¹. This study clearly demonstrated that Giemsa C-banding patterns contribute significantly to infrasubgeneric grouping and generic demarcation, and that the so-called "banding style" may cast new light on evolutionary relationships both within and between the groups.

Rix² and Rechinger³ have classified all Iranian species of *Fritillaria*⁴ in four subgenera: *Fritillaria*, *Theresia*, *Petilium* and *Rhinopetalum*, of which *Rhinopetalum* is now accepted as a distinct genus. *Fritillaria* is morphologically divided into three species, *F. crassifolia*, *F. kotschyana* and *F. caucasica*. *F. crassifolia* and *F. kotschyana* show very similar flowers and leaf morphology. They can be divided into two complexes based on nectary characters⁴. *F. caucasica* shows compara-

सारांश

ईरान की फ्रिटिलेरिया स्पीसीज समूह की तीन प्रजातियों, फ्रिटिलेरिया काकेशिया (एडेम्स), फ्रिटिलेरिया उवा-वुल्पिस (रिक्स) और फ्रिटिलेरिया एसीरियाका (बेकर) के सी-पट्टों के नमूनों तथा दूसरे गुणसूत्री लक्षणों का वर्णन किया गया है। इन सभी प्रजातियों के मूल गुण-सूत्र ($n=12$) एक जैसे हैं, जिनमें बड़े सुडौल तथा छोटे बेडौल गुण सूत्र हैं परन्तु इनके बीच के सी-पट्टे विभिन्नता दर्शाते हैं। ये पट्टे सेन्ट्रोमेरिक, अंतर्वेशी तथा टेलोमेरिक स्थानों पर अनुपूरक सिलवटों के सन्निकट स्थित हैं। फ्रिटिलेरिया काकेशिया तथा फ्रिटिलेरिया एसीरियाका दूसरों से एक सब मेटासेन्ट्रिक गुणसूत्री जोड़े की उपस्थिति की विभिन्नता दर्शाते हैं। इसके अतिरिक्त मूलभूत हेट्रोक्रोमेटिन, हेट्रोमार्फिज्म, समस्थानिक सी-पट्टों का वितरण, सी-पट्टों का विकासक्रम तथा सी-पट्टों की वर्गीकरण में भूमिका का भी पर्यालोचन किया गया है।

सांकेतिक शब्द : फ्रिटिलेरिया स्पीसीज समूह, सी-पट्टी, मूलगुण सूत्र, हेट्रोक्रोमेटिन

tively small and narrowly campanulate flowers, and the nectaries are usually placed either at the base or only 0.5-1 (-2) mm above the base of the perianth.

Earlier studies of the distribution of constitutive heterochromatin in chromosomes of some Iranian species belonging to genus *Fritillaria* showed that the incidence and pattern of C-bands in this group may throw some light on evolution and speciation within the genus⁵.⁶ Unfortunately the whole material stemmed from a horticultural collection with no information regarding exact origin or number of populations.

The aim of the present investigation is to give a general over view of the variations of C-banding patterns found in the Iranian *Fritillaria* spp. group to provide data useful for phylogenetic and cytotaxonomic works.

Material and Methods

The specimens for this study were collected by the author from different localities in Iran (Table 1),

except *F. uva-vulpis* which was received from some other source. The bulbs collected were cultivated under uniform conditions in clay pots in indoor beds, in order to check the morphological characters and also for root tip preparations for chromosomal studies. Each population was assigned a collection number preceded by the abbreviation GBK which stands for the author's name. For chromosome studies, root tip meristem was used. The roots were treated with a mixture of 0.2% colchicine and 0.002 M 8-hydroxyquinoline (1 vol. each) for 2-3 hours at room temperature, fixed in Carnoy's fixative (ethanol/acetic acid 3:1) for 24 hours, transferred to 70% alcohol and stored at -20°C until used. Fixed material was washed in distilled water and hydrolysed in 1 M HCl for 15 min. at room temperature. After another rinse, they were softened in a mixture of 10%-20% pectinase (Sigma) dissolved in 40% glycerol and 1% cellulase (Calbiochem) (1 vol. each) at 37°C for 1 hour. For acetic orcein staining, only 5% powdered pectinase for 2-3 hours at room temperature was used.

The meristematic tip of the root was separated and squashed in 45% acetic acid on a clean glass slide. Coverslips were removed after freezing in liquid nitrogen and slides immersed in 95% ethanol, dried in compressed air and stored in an exsiccator for a week. Dried slides were treated in freshly prepared 5% Barium hydroxide [Ba(OH)₂] at 50°C-52°C for 7 min., washed in running tap water for 1 hour and immersed in 2 x SSC (pH=7.0) at 60°C for 60 min. After a rinse in Sørensen's phosphate buffer (pH 6.81), cells were stained in 1.5% Giemsa (buffered to pH 6.81) for 5-8 min., rinsed, dried, and mounted in D.P.X. Karyograms were prepared from drawings of metaphase chromosomes by using camera lucida.

Karyotypes were characterized by 1) pairing⁷ i.e. indicating the pairs of homologous chromosomes in decreasing order of the length 2) typifying the kind of chromosome, according to centromere position 3) ratio of long arm to short arm (L:S)^{6, 7, 8, 9, 22}. All measurements and percentage values given in the tables are mean values from 10 plates from a representative population.

Results

The somatic chromosome number (2n) was 24 for all species studied. All of the populations listed in Table 1 were found to have C-bands in their

chromosome complements, some more than the others. The banding patterns are presented in the form of idiograms (Fig. 2). Based upon the intensity of the stain and the constancy of the banding, these bands were divided into two main categories, namely major and minor bands. The major bands (solid black and stippled areas in idiograms) stained very darkly, could be observed in every cell and were apparent from prophase to the highly contracted metaphase chromosomes. The minor bands comprised two classes: 1) lightly stained bands (broken lines in the idiograms) which varied in intensity and appearance from cell to cell, and 2) dot-like bands (double dots in idiograms) which were irregularly present and mostly located at intercalary positions. The bands are further divided into four groups according to their position: centromeric bands situated at one or both sides of the centromere, intercalary bands, telomeric bands (at the chromosome ends), and bands adjacent to the secondary constriction in the short arm of the satellited chromosomes. Apart from these, in some species the short arms of some chromosomes are entirely heterochromatic. Summary of karyotype characters of the species studied are listed in Table 5 and the percentage of heterochromatin in the haploid complement of the species studied is presented in the Tables 5 and 6.

(1) *F. caucasica* Adams. A Giemsa C-banded plate is shown in Fig. 1 a. Some important particulars of the karyotype are listed in Table 2. The karyotype consists of two metacentric, one submetacentric, four subtelocentric and five telocentric chromosome pairs (Fig. 2a). The pair no. 3 was found to have a small satellite in the short arm. The Giemsa C-banding pattern is characterized by the following: Five centromeric bands exist in the short arms of chromosome pairs nos. 6, 8, and 10, and in the long arm of pair nos. 7 and 11; major intercalary bands are absent in the short arms, but present in the long arms of pair nos. 1, 3, 5, 9, 10 and 12; telomeric bands occur in the short arms of pair nos. 2 and 4, and in the long arm of pair no. 1; a band occurs adjacent to the secondary constriction in the short arm of pair no. 3. Apart from these, there are many minor bands irregularly distributed along the chromosome arms. Heteromorphy in band size was evident in the long arms of pairs nos. 1, 3 and 7. The karyotype formula is $2n=24=4m + 2sm + 8st + 10t$.

(2) *F. uva-vulpis* Rix. A Giemsa C-banded plate is shown in Fig. 1b. Some particulars of the karyotype are listed in Table 3. The karyotype contains two

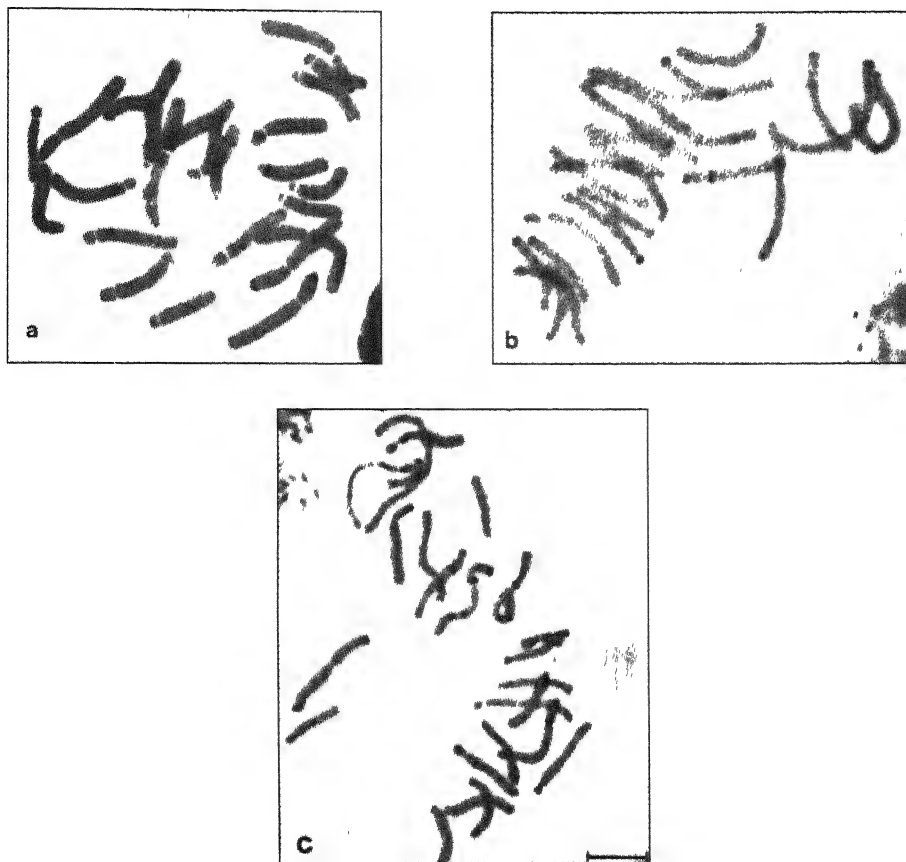


Fig. 1 - Giemsa C-banded metaphase of the species studied belonging to *Fritillaria* group, a *F. caucasica* ($2n=24$), Population GBK 70; b *F. uva-vulpis* ($2n=24$), Population TUBERGEN from Iraq; c *F. assyriaca* ($2n=24$), Population SÖNDERHOUSEN 1106 from NE Turkey. - Scale: 10 μ m.



Fig. 2 - Haploid idiograms of the species studied belonging to *Fritillaria* group, showing interspecific variation in karyotype. band size and numbers, a *F. caucasica*, b *F. uva-vulpis*, c *F. assyriaca*. Stipple areas: heteromorphic bands; double dots: dot-like "bands"; broken lines: thin pale bands. - Scale: 10 μ m.

metacentric, eight subtelocentric and two telocentric chromosome pairs (Fig. 2b). Unlike *F. caucasica*, a pair of submetacentric chromosomes is absent. Of major C-bands there are eight centromeric bands present, five of which occur in the short arms of pairs nos. 4–5, 8–9 and 10, and three in the long arms of pairs nos. 3–4 and 8; distinct intercalary bands exist in the short arm of pair no. 1, and in the long arms of pairs nos. 1, 3, 5–6, 8 and 12; telomeric bands occur in the short arms of pairs nos. 2–3, 7, 9 (the short arm of pair no. 9 was entirely heterochromatic), and in the long arms of pairs nos. 1, 3, 5 and 11 no secondary constrictions were found. Apart from these and in accordance with the other species, chromosomes of *F. uva-vulpis* have some additional thin, pale and dot-like bands at intercalary positions. Band heteromorphy with respect to size was observed in chromosome pair nos. 1–2 and 5. The karyotype formula is $2n=24=4m+16st+4t$.

(3) *F. assyriaca* Baker. A Giemsa C-banded plate is shown in Fig. 1c. Some important particulars of the karyotype are listed in Table 4. The karyotype consists of two metacentric, one submetacentric, five subtelocentric and four telocentric chromosome pairs (Fig. 2c). Distinct centromeric C-bands are seen in the short arms of pairs nos. 2, 4, 7, and 9, and in the long arms of pairs nos. 6 and 11; major intercalary bands occur in the short arms of pairs nos. 1–2, 5, and in the long arms of pairs nos. 2–5, 7–8 and 12; telomeric bands are present in the short arms of pairs nos. 1–3, 6, and 10, and in the long arms of pairs nos. 1, 6, and 11; the short arm of the SAT-chromosome pair no. 7 was, except for the satellite, entirely heterochromatic. In addition to these major bands, there are some minor bands in the karyotype. Heteromorphy with respect to band size was evident in the chromosome pairs nos. 1–3, 6 and 12. A pair of submetacentric chromosomes is present in *F. assyriaca* as in case of *F. caucasica*. The karyotype formula is $2n=24=4m+2sm+10st+8t$.

Discussion

The present observations show that three species of *Fritillaria* present C-bands, some more than the others (Table 5, 6). The chromosome number determinations of $2n=24$ for *F. caucasica* and *F. assyriaca* are new reports for the geographic zones from which these plants were collected. However, they agree with determinations based on material of these taxa from

other areas^{5, 6}. The somatic chromosome number of $2n=24$ for *F. uva-vulpis* is reported here for the first time.

In general, it seems that the amount of constitutive heterochromatin is relatively higher in species belonging to the subgenus *Fritillaria* than those of the subgenera *Petilium* and *Theresia*⁵. Also, La Cour^{5, 6, 8} believed that Old World species of *Fritillaria* usually have less heterochromatin than those of New World origin. One point perhaps worthy of consideration, particularly since there is little interspecific hybridization in fritillaries, is that the American fritillaries, with an abundance of heterochromatin, may possibly be relics. The forest or forest border habitats in north-west California where most of the American fritillaries occur have many tertiary relics. The climate of the area has been favourable for long-time survival, and the rocky serpentine type of terrain may have enabled some to survive free of competition. The evaluation of these findings is, however, a matter for the future, as the understanding of the evolution of heterochromatin is in many ways, far from complete.

There seems to be little doubt from the present observations that at least two classes of heterochromatin occur in *Fritillaria*. These are one represented by intensely stained and relatively thick (major) bands, the other by weakly stained, thin C-bands and/or dot-like bands (minor bands). Both classes have been previously recorded in *F. crassifolia*^{5, 6, 9}. It is assumed that the pale bands and the dot-like bands are part of one and the same phenomenon and one which is most simply explained in terms of packing of the chromatin involved. Accordingly, it is believed that the two classes of heterochromatin diverge in respect to their degree of condensation. It may well be, as the present observations of interphase, and also previous findings in *Fritillaria* suggest, that the distinction exists for the whole of the mitotic cycle. The position of such bands in metaphase chromosomes has been demonstrated to correspond exactly with that of H-segments resolved as weakly stained regions in Feulgen squashes of roots from plants grown at low temperatures¹⁰. In a comparative study of *Fritillaria* and *Scilla*, the findings in *Fritillaria* were in marked contrast to those obtained in roots of *Scilla sibirica*^{11, 12}. The H-segments of this plant could not be identified by the same criteria, although it is well-known that C-bands are demonstrable in its chromosomes with the Giemsa technique. Thus even at the time of these

Table 1 - Original sources of material studied of *Fritillaria* spp. group.

Pop. no.	species	Locality
GBK 70	<i>F. caucasica</i>	Iran: E. Azarbaijan, road from Tabriz to Ahar, steep-stony slopes, 1800-2150 m
TUBERGEN s.n	<i>F. uva-vulpis</i> *	Iraq: Rowandooz, Haji Omran.
SØNDERHOUSEN	<i>F. assyriaca</i> *	Turkey: Agri, Tahir Pa. pass.

* Material for karyological studies obtained from cultivated plants in the Goteborg Botanical Garden, Sweden.

Table 2 - Details of karyotype in *F. caucasica* (GBK 70).

Pair	L+S(μm)	L(μm)	S(μm)	L+S%	L%	S%	L/S	Type
1	18.18	10.83	7.35	11.86	7.05	4.81	1.47	m
2	17.83	11.06	6.77	11.51	7.14	4.37	1.63	m
3	14.41	9.74	4.67	9.29	6.28	3.01	2.08	sm
4	13.88	12.72	1.16	8.96	8.21	0.75	10.94	t
5	13.23	11.29	1.94	8.54	7.29	1.25	5.83	st
6	12.45	11.16	1.29	8.03	7.20	0.83	8.67	t
7	12.07	11.09	0.98	7.78	7.15	0.63	11.34	t
8	11.42	10.44	0.98	7.37	6.74	0.63	10.69	t
9	11.34	9.66	1.68	7.31	6.23	1.08	5.76	st
10	10.76	8.93	1.74	6.88	5.76	1.12	5.14	st
11	10.12	8.10	2.02	6.53	5.23	1.30	4.02	st
12	9.21	8.18	1.03	5.94	5.27	0.67	7.86	t

studies, it was apparent that more than one class of heterochromatin was present in chromosomes of plants, corresponding to different degrees of condensation. In three *Fritillaria* species presently studied, different numbers of dot-like bands could be recognized in the chromosomes. These were mostly intercalary and partly centromerically positioned. Small centromeric dots were first demonstrated by Eiberg¹² in Giemsa preparations of human chromosomes.

Just as in animals, there is extensive variation of heterochromatin in plants. Most of the work on heteromorphism in plants has been based on C-banding which shows many interesting features. In particular, it has been possible in some species to make correlations with the phenotype of the plants. A number of examples of heteromorphism of C-bands in plants could be mentioned. For example, the chromosomes of *Scilla sibirica* show extensive heteromorphism in their heterochromatin, which is both C-banded and intensely

fluorescent with Hoechst 33258. In several species of *Allium*, Vosa^{13, 14} also described a number of different classes of heterochromatin, according to whether or not they were C-banded, and whether they showed increased or decreased fluorescence or no differentiation with Quinacrine or Hoechst 33258. The heterochromatic segments varied in number and size, and were reported to replace or possibly to add to, normal euchromatic segments. Marks and Schweizer¹⁵ described heteromorphism for both number and size of bands in *Anemone*, while Filion¹⁶ found large differences in banding patterns between different cultivars of *Tulipa*. Bentzer and Landstrom¹⁷ described a homologous pair of chromosomes in *Leopoldia comosa* which exhibited between three and eight C-bands, and which also varied in width and staining intensity. Heteromorphy in *Fritillaria* and *Rhinopetalum* was also previously reported^{1, 5, 6}. In addition to polymorphism in general chromosome morphology in *Fritillaria*, just as in *Tulipa* there is evidently also heteromorphy of

Table 3 - Details of karyotype in *F. uva-vulpis* (TUBERGEN from Iraq)

Pair	L+S(μm)	L(μm)	S(μm)	L+S%	L%	S%	L/S	Type
1	19.28	12.01	7.27	11.28	7.02	4.26	1.65	m
2	18.16	11.58	6.58	10.55	6.73	3.82	1.76	m
3	17.13	14.80	2.33	9.95	8.60	1.35	6.37	st
4	15.83	13.63	2.20	9.20	7.92	1.28	6.18	st
5	14.67	12.80	1.87	8.52	7.44	1.08	6.88	st
6	14.01	12.20	1.81	8.14	7.09	1.05	6.75	st
7	13.38	11.42	1.96	7.78	6.64	1.14	5.82	st
8	12.57	11.16	1.41	7.31	6.49	0.82	7.91	t
9	12.39	11.06	1.33	7.20	6.43	0.77	8.35	t
10	12.07	10.26	1.81	7.01	5.96	1.05	5.67	st
11	11.51	9.92	1.59	6.72	5.76	0.96	6.00	st
12	10.89	9.43	1.46	6.33	5.48	0.85	6.44	st

Table 4 - Details of karyotype in *F. assyriaca* (SPENDERHOUSEN 1106).

Pair	L+S(μm)	L(μm)	S(μm)	L+S %	L%	S%	L/S	Type
1	19.82	11.22	8.60	11.79	6.67	5.12	1.30	m
2	18.30	11.55	6.75	10.82	6.83	3.99	1.71	m
3	16.95	11.42	5.53	10.02	6.75	3.27	2.06	sm
4	16.44	14.65	1.79	9.71	8.66	1.05	8.16	t
5	14.88	12.96	1.92	8.79	7.66	1.13	6.77	st
6	13.60	11.53	2.07	8.04	6.81	1.23	5.53	st
7	13.03	11.68	1.35	7.69	6.90	0.79	8.73	t
8	12.20	10.52	1.68	7.21	6.22	0.99	6.28	st
9	11.94	10.72	1.22	7.06	6.34	0.72	8.80	t
10	11.42	9.87	1.55	6.74	5.83	0.91	6.40	st
11	10.92	9.03	1.89	6.45	5.34	1.11	4.81	st
12	9.60	8.46	1.14	5.67	5.00	0.67	7.46	t

bands. In agreement with conditions in other related genera, three types of band heteromorphy were encountered in the present investigation. Firstly, a heteromorphy occurs in size of a particular band in a homologous pair of chromosomes. This was mostly common and very distinct above all in meta- and submetacentric chromosome pairs of all species examined (Fig. 2, stippled areas). Secondly, heteromorphy

may manifest itself in the presence or apparent absence of a particular band.

Heterochromatic segments also varied in amount, number and position between different species. It is apparent from this study that wide differences exist between fritillaries in the distribution and amount of detectable heterochromatin contained in their chromo-

Table 5 - Summary of karyotype characters of the *Fritillaria* species studied.

Species	Range of chromosome length (μm)	Total haploid karyotype length (μm)	Karyotype formula
<i>F. caucasica</i>	9.21-18.18	154.20	2n=24=4m + 2sm+8st+ 10t
<i>F. uva-vulpis</i>	10.89-19.28	171.89	2n=24=4m + 16st + 4t
<i>F. assyriaca</i>	9.60-19.82	169.10	2n=24=4m + 2sm+ 10st + 8t

Table 6 - Proportion (in %) of Giemsa C-bands to chromosome length.

Note: dot-like and thin pale bands (presented by double dots and broken lines in idiograms) are not considered in this calculation

Species	Pop. no.	Chromosome no.												% of heterochromatin in haploid complement
		1	2	3	4	5	6	7	8	9	10	11	12	
<i>F. caucasica</i>	GBK 70	3.0	1.8	3.0	1.7	0.3	1.8	1.3	1.7	1.0	2.3	1.7	0.4	3.80
<i>F. uva-vulpis</i>	TUBERGEN	6.1	2.3	3.5	2.0	4.4	1.8	1.0	4.4	5.1	0.0	1.5	0.8	4.96
<i>F. assyriaca</i>	SØNDERHOUSEN	6.7	6.6	3.0	4.7	2.0	3.9	5.9	4.2	2.3	1.5	1.4	1.5	6.70

somes. A summary of heteromorphy, and number and distribution of bands is presented in Table 7. Heteromorphism is likely to be very much involved in the variation in total DNA connected to specific evolution. The set of bands revealed by C-banding procedures usually consists of different forms of heterochromatin¹⁸, and intraspecific variation in C-banding heteromorphy must be largely due to differences of amounts and kinds of constitutive heterochromatin coupled with variation of chromosome contraction.

It seems that C-band material and heterochromatin in general are relatively plastic nuclear components which may be subjected to considerable quantitative and qualitative changes during karyotype evolution. C-band negative (and generally euchromatic) chromosome segments, on the other hand, may exhibit higher degrees of conservation. This phenomenon is already well known for plants and has also become particularly evident in certain mammalian systems where specific staining reactions such as Q- or G-banding also allows for discrimination of other chromosome segments than C-bands. A study on species of the American rodent genus *Peromyscus* showed that karyotype differences between the two species *P. crinitus* (2n=48, 56 chromosome arms per diploid complement) and *P. boyleyi* (2n=48, 96 chromosome arms) are solely due to differences in C-band content: the second arms, which differ in number, are totally heterochromatic in both species whereas the G-banding patterns of the C-negative arms

were identical (Arrighi *et al.*)¹⁹. According to La Cour^{5,6} out of thirty-seven species of *Fritillaria*, examined twelve out of twenty-seven Old World species and seven out of nine New World species had detectable heterochromatin. It was concluded that New World species overall have an appreciably higher content of heterochromatin. So, it is reasonable to assume that the direction of evolution in *Fritillaria* is towards heterochromatin increase. It is also clear from the present investigation that wide differences exist between fritillaries in the distribution and amount of heterochromatin even between Old World species. What does this mean in terms of evolution? The answer may possibly lie in the directed evolution of DNA with highly repetitive sequences, the most likely component of constitutive heterochromatin. A correlation between highly repeated satellite DNA and C-banding positive heterochromatin was demonstrated in *Scilla siberica* (Timmis *et al.*)²⁰. For highly reiterated simple sequence DNA, some models of evolution have been proposed and it was suggested that highly repeated DNA, such as satellite DNA, is evolving more rapidly than unique sequence DNA. If the above correlation between highly repeated DNA and C-banding is generally true also for plant chromosomes, it may be possible to draw analogous lines for the evolutionary trends of C-band material in plants.

A model which attempts to explain the concept of equilocality (Schweizer and Loidl)²¹ assumes that telomeres (including centric regions in the aero-telocentric

chromosomes) are C-band initiation sites. These regions are thus presumptive sites where heterochromatin amplification tends to be initiated, and from which heterochromatin sequences are dispersed. Transposition may be coupled with the amplification process. Transpositional spread requires proximity and/or physical contact. All the potential contact zones which lie at a similar distance from the centromere (i.e. equilocal sites) are potential sites of heterochromatin transfer from telomere to intercalary locations during evolutionary time period. This model of band distribution predicts that karyotypes consisting of chromosomes with approximately similar arm length will tend to have telomeric bands rather than intercalary bands, and a karyotype consisting of members with different arm ratios, will also tend to possess intercalary bands in addition to telomeric bands. It is very clear from the present investigation, that there is great diversity of arm ratios (L/S in Tables 2-4) in the karyotype of the *Fritillaria*

species examined, and not surprisingly, in addition to telomeric bands, there are also many intercalary bands in the chromosomes.

The C-banding pattern is the existence of one common, and \pm similar model of banding in a group or groups of plants is often useful in taxonomy. Banding patterns can be used to study chromosomal changes that have occurred between related species. In many cases differences in banding patterns between related species are very small, although complete identity is comparatively rare. More often, some differences can be found even between closely related species. Changes in the amount of heterochromatin, demonstrated by C-banding, are quite common. Several C-banding studies have attempted to recognise ancestral genomes by heterochromatin markers, especially in allopolyploids²². As regards taxonomic applications of C-banding, Marks and Schweizer (1974)¹⁵ were the first to demonstrate

Table 7 - Frequency and distribution of C-bands and heteromorph bands in the chromosomes of material studied of *Fritillaria* spp. group. Numerator: number of positions with heteromorph bands; denominator total number of positions with bands. Note. dot-like and thin pale bands (presented by double dots and broken lines in idiograms) are not considered in this table.

Species	Band position	Chromosome no.											
		1	2	3	4	5	6	7	8	9	10	11	12
<i>F. caucasica</i>	Intercalary	2/2	0/0	1/1	0/0	0/1	0/1	0/0	0/0	0/1	0/1	0/0	0/1
	Centromeric	0/0	0/0	0/2	0/1	0/2	0/1	0/1	0/1	0/0	0/1	0/1	0/0
	Telomeric	0/1	0/1	0/0	0/1	0/0	0/0	0/0	0/0	0/0	0/0	0/0	1/0
	Sec constriction	0/0	0/0	0/1	0/0	0/0	0/1	0/0	0/0	0/0	0/0	0/0	0/0
<i>F. uva-vulpis</i>	Entirely	0/0	0/0	0/0	0/0	0/0	0/0	0/0	0/0	0/0	0/0	0/0	0/0
	heterochromatic short arm Intercalary	2/4	0/0	0/1	0/0	0/1	0/2	0/0	0/2	0/0	0/0	0/0	0/1
	Centromeric	0/0	0/0	0/1	0/2	0/1	0/0	0/0	0/2	0/1	0/1	0/0	0/0
	Telomeric	0/1	1/1	0/2	0/0	0/1	0/0	0/1	0/0	0/1	0/0	0/1	0/0
	Sec. constriction	0/0	0/0	0/0	0/0	0/0	0/0	0/0	0/0	0/0	0/0	0/0	0/0
<i>F. assyriaca</i>	Entirely	0/0	0/0	0/0	0/0	0/0	0/0	0/0	0/0	0/1	0/0	0/0	0/0
	heterochromatic short arm Intercalary	1/2	1/2	1/1	0/3	0/3	0/0	0/1	0/3	0/0	0/0	0/0	1/1
	Centromeric	0/0	0/1	0/0	0/1	0/0	1/1	0/1	0/0	0/1	0/0	0/1	0/0
	Telomeric	1/2	1/1	0/1	0/0	0/0	0/2	0/0	0/0	0/0	0/1	0/1	0/0
	Sec. constriction	0/0	0/0	0/0	0/0	0/0	0/0	0/1	0/0	0/0	0/0	0/0	0/0
	Entirely heterochromatic short arm	0/0	0/0	0/0	0/0	0/0	0/0	0/1	0/0	0/0	0/0	0/0	0/0

the impact that banding of *Anemone* and *Hepatica nobilis* would have similar significance in plant systematics. C-banded karyotypes of \pm related taxa indicated a relatively high degree of karyomorphological differentiation in some woody species of *Liriodendron* (Magnoliaceae,) and the results were considered in karyosystematic comparisons²³. On the basis of C-banding, *Artemisia* (Compositae, Anthemideae) is characterized as a genus with few intercalary C-bands in chromosomes²² and this result was in agreement with the results of Schweizer and Ehrendorfer²⁴, who found this pattern to be common in the tribe Anthemideae. A comparison between the C-banded karyotypes of two species of *Festucopsis* (Poaceae, Triticeae) supported their systematic position in two genera^{24, 25}.

Surveys of bulbous genera such as *Leopoldia* (Bentzer)^{26, 27, 28}, *Allium* (Vosa)^{13, 14}, *Fritillaria* (La Cour)^{1, 5, 6}, *Tulipa* (Blakey and Vosa)²⁹, *Scilla* (Greilhuber)^{10, 11} and *Lilium* (Smith *et al*)³⁰ have been given. All these studies show that a large amount of variation in heterochromatin amount exists within each genus, and chromosome banding results usually correspond to existing taxonomic groupings, although sometimes some disagreements or within-group heterogeneities were noted.

In *Fritillaria* s.lat, according to Bakshi Khaniki¹ large differences exist in the amount and distribution of heterochromatin, not only between groups but also within them, the divergence being extreme in the American subgenus *Liliorhiza*, or within the *Fritillaria* group. In the present investigation, the chromosome banding results, for *Fritillaria* subgen. *Petilium* and *Theresia* corresponded to existing morphologically based taxonomic grouping. In the genus *Rhinopetalum*, the closely related *R. gibbosum* and *R. arianum* present almost identical banding patterns. Presence of a centromeric band in the short arm of the second pair of m-chromosomes in *R. bucharicum* distinguishes it from the other two species. These C-banding results confirm the morphological grouping of these *Rhinopetalum* species into two subgroups, based on nectary morphology⁴.

It is also evident from the present investigation that certain differences exist in the amount and distribution of C-bands between closely related species. There is much divergence in C-banding pattern between the Iranian species of *Fritillaria* spp. group, of which *F. caucasica* and *F. assyriaca* possess a pair of submetacentric chro-

mosomes with telomeric and intercalary bands. Apart from this, a pair of SAT-chromosome was observed in both species. There are many intermediates between *F. caucasica* and other species, particularly *F. assyriaca*, in the group in NE Turkey. Both species are decidedly similar in some morphologic characters and may be considered as a variant of one species. Similar banding results may confirm this suggestion. *F. chlorantha* presented a comparatively high amount of heterochromatin, mostly distributed at centromeric locations. Furthermore, the short arms of pairs nos. 5 and 11-12 were entirely heterochromatic. This species is morphologically similar to *F. atrolineata*² mainly in the colour of its flowers, but exhibits a different C-banding pattern. *F. zagrica* is morphologically unique by having the dark purple flowers adorned with a bright yellow apex. It was the only species in this group which presented accessory B-chromosomes¹. It is evident here that large differences exist in banding patterns in species of the *Fritillaria* spp. group, and it is difficult to formulate a basic banding pattern to confirm the taxonomic grouping, though the latter is well documented by morphologic characters. The C-banding results for Iranian species belonging to the *Fritillaria* spp. group is in agreement with that of La Cour^{5, 6, 8} on European species for the same group. Congruence of taxonomic grouping with similarity in chromosome structure is not expected to occur generally even in an ideally perfect system¹.

This survey has shown that C-band patterns can often be useful in deducing relationships between species belonging to *Fritillaria* spp. group, and that they are also useful for species separation.

References

1. Bakshi Khaniki G. Taxonomy and karyology of the genus *Fritillaria* s.lat (*Liliaceae*) in Iran with special reference to the species in Iran. Ph.D Thesis Gothenburg University, Sweden, 1998
2. Rix EM *Fritillaria* (*Liliaceae*) in Iran. *Iranian J Bot* 1997; 1 : 751-755.
3. Rechinger KH *Fritillaria* Flora Iranica vol 165 Graz Akademische Druck-u-Verlagsanstalt, 1990.
4. Bakshi Khaniki G, Persson K. Nectary morphology in South West Asian *Fritillaria* (*Liliaceae*) *Nordic J Bot* 1997; 17 : 579-587
5. La Cour LF Two types of constitutive heterochromatin in the chromosomes of some *Fritillaria* species. *Chromosoma* 1978; 67 : 67-73

6. La Cour LF. The constitutive heterochromatin in chromosomes of *Fritillaria* sp. as revealed by Giemsa banding. *Philos Tran Roy Soc London ser B* 1978; **285** : 61-68.
7. Levan A, Fredga K, Sandberg S. Nomenclature for centromeric position on chromosomes. *Hereditas* 1965; **52**: 201-210.
8. La Cour LF. Heterochromatin and the organization of nucleoli in plants. *Heredity* 1951; **5** : 231-239.
9. La Cour LF. Differential Giemsa staining of B-chromosomes of *Fritillaria tenella*. *Heredity* 1978; **41** : 101-103.
10. Greilhuber J. Chromosomal evidence in taxonomy. In : Heywood VII, Moore DM, editors. Current concepts in plant taxonomy. London : Academic Press. 1984.
11. Greilhuber J. Evolutionary aspects of chromosome banding, heterochromatin, satellite DNA, and genome size in *Scilla* (Liliaceae). *Berlin Deutsch Bot Ges* 1981; **94** : 249-266.
12. Vosa CG. Heterochromatin recognition and analysis of chromosome variation in *Scilla sibirica*. *Chromosoma* 1973; **43** : 269-278.
13. Vosa CG. Heterochromatic banding patterns in *Allium*. I. The relationship between the species of the cepa group and its allies. *Heredity* 1976; **361** : 383-392.
14. Vosa CG. Heterochromatic banding patterns in *Allium*. II. Heterochromatin variation in the species of the *paniculatum* group. *Chromosoma* 1976; **57** : 119-133.
15. Marks GE, Schweizer D. Giemsa banding: karyotype differences in some species of *Anemone* and in *Hepatica nobilis*. *Chromosoma* 1974; **44** : 405-416.
16. Filion WG. Differential Giemsa staining in plants. I. Banding patterns in three cultivars of *Tulipa*. *Chromosoma* 1974; **49** : 51-60.
17. Bentzer B, Landstrom T. Polymorphism in chromosomes of *Leopoldia comosa* (Liliaceae) revealed by Giemsa staining. *Hereditas* 1975; **80** : 219-232.
18. Sumner AT. Chromosome banding, London : Unwin Hyman. 1990.
19. Arrighi FE, Stock AD, Pathak S. Chromosomes of *Peromyscus* (Rodentia, Cricetidae). V. Evidence of pericentric inversions. *Chromosomes Today* 1976; **5** : 323-329.
20. Timmis JN, Deumling B, Ingle J. Localisation of satellite DNA sequences in nuclei and chromosomes of two plants. *Nature* 1975; **257** : 152-155.
21. Schweizer D, Loidl J. A model for heterochromatin dispersion and the evolution of C-bands pattern. *Chromosomes Today* 1987; **9** : 61-74.
22. Oliva M, Valles J. Karyological studies in some taxa of the genus *Artemisia* (Asteraceae). *Canadian J Bot* 1994; **72** : 1126-1135.
23. Morawetz W. C-banding in *Liriodendron tulipifera* (Magnoliaceae) : Some karyological and systematic implications. *Plant Syst Evol* 1981; **138** : 209-216.
24. Schweizer D, Ehrendorfer F. Giemsa banded karyotypes, systematics, and evolution in *Anacyclm* (Asteraceae-Anthemideae). *Plant Syst Evol* 1976; **126** : 107-148.
25. Linde-Laursen I, Seberg O, Frederiksen S, Baden C. The karyotype of *Festucopsis serpentini* (Poaceae-Triticeae) from Albania studied by banding techniques and in situ hybridization. *Plant Syst Evol* 1976; **201** : 75-82.
26. Bentzer B. Variation in the chromosome complement of *Leopoldia comosa* (L.) Parl. (Liliaceae) in the Aegean (Greece). *Botanical Not* 1972; **125** : 406-418.
27. Bentzer B. Taxonomy, variation and evolution in representatives of *Leopoldia* Parl. (Liliaceae) in the southern and central Aegean. *Botanical Not* 1973; **126** : 69-132.
28. Bentzer B, Landstrom T. Polymorphism in chromosomes of *Leopoldia comosa* (Liliaceae) revealed by Giemsa staining. *Hereditas* 1975; **80** : 219-232.
29. Blakey DH, Vosa CG. Heterochromatin and chromosome variation in cultivated species of *Tulipa* subgen. *Leiostemones* (Liliaceae). *Plant Syst Evol* 1982; **139** : 163-178.
30. Smyth DR, Kongsuwan K, Wisudharomn S. A survey of C-band patterns in chromosomes of *Lilium* (Liliaceae). *Plant Syst Evol* 1989; **163** : 53-69.

Studies on the diversity and pattern of vertical distribution of some epiphytic pteridophytes on their host plants of southern Sikkim, India

GAUTAM GANGULY and RADHANATH MUKHOPADHYAY*

Pteridology Laboratory, Department of Botany, The University of Burdwan, Burdwan-713104, India.

*e-mail : rnm13351@yahoo.com

Received December 26, 2006, Revised April 4, 2007, Accepted April 10, 2007

Abstract

The objectives of the present work were to determine the epiphytic pteridophyte composition, pattern of vertical distribution and diversity of epiphytic pteridophytes on the host trees of Sikkim area as well as to demarcate the 'specialist species' (species having restricted vertical distribution). The occurrence of epiphytic pteridophytes in relation to the type of forest, altitude and environmental factors like rainfall, relative humidity and temperature have also been determined. It was found that the upper hill forest zone harbours the highest diversity of epiphytic pteridophytes. The richness of epiphytic pteridophytes was maximum in the upper foot of the host plants, while it was minimum in the tree top region. *Pyrrosia lanceolata* (L.) Farwell, *Oleandra wallichii* (Hook.) Pr., *Polypodioides lachnopus* (Wall. ex Hook.) Ching and *Arthromeris walttchiana* (Spreng.) Ching were found to have wide vertical distributions. While, *Pseudodrynaria coronans* (Wall. ex Hook.) Ching, *Microsorium punctatum* (L.) Copel., *Nephrolepis cordifolia* (L.) Pr., *Lepisorus ussuriensis* (Regel et Maack) Ching, *Polypodiastrium argutum* (Wall. ex Hook.) Ching, *Pyrrosia manii* (Gies.) Ching, *Selaginella involvens* (Sw.) Spring and *Ctenopteris subfalcata* Blume ex Kunze have restricted vertical distribution on the host plants. Specialist species vary with the altitude, except two ferns and the generalist species were more or less common in different altitudinal zones.

Key words : vertical distribution, epiphyte diversity, specialist species, generalist species, conservation, Sikkim.

Introduction

The altitude of Sikkim varies from 224 m to 8500 m. The climate and vegetation vary with the change in altitude. Altitude-wise vegetation of this state is divided into five zones¹. These are—

1. Low hill forest (up to 750 m).
2. Middle hill forests (750-1500m).

सारांश

सिक्किम के पोषी वृक्षों पर ऐसे टेरिडोफाइट पौधों, जो अधिपादप हो, की सरचना, खड़ी विस्तारिता तथा विभिन्नता का अध्ययन, साथ ही साथ विशेषज्ञ प्रजातियों (जो सीमित खड़ी विस्तारिता दर्शाती हों) का निर्धारण, वर्तमान कार्य का उद्देश्य है। इसके अतिरिक्त इस प्रकार के टेरिडोफाइट किस प्रकार के वनों में पाये जाते हैं, कितनी ऊँचाई पर पाये जाते हैं तथा वातावरणीय कारकों जैसे वर्षा, तुलनात्मक आद्रता तथा तापमान का इनके उपस्थिति पर क्या प्रभाव पड़ता है, इन सभी तथ्यों का भी अध्ययन किया गया है। अधिपादप प्रकृति के टेरिडोफाइट पौधों की अत्यधिक विभिन्नता ऊपरी पहाड़ी वन क्षेत्र में मिली है। इस प्रकार के टेरिडोफाइट पौधों की अधिकतम प्रचुरता जहाँ पोषी पौधों के तने के ऊपरी हिस्से में दिखती है, वही न्यूनतम प्रचुरता पेड़ के ऊपरी हिस्से में दिखती है। पाइरोसिया लैन्सियोलेटा (लीनियस) फारवेल, ओलीएन्ड्रा वालिची (हुक) प्र०, पालिपोडायोयडिस लैचनोपस (वाल एक्स हुक) चिंग और आर्थ्रोमेरिस वेलीचियाना (स्प्रेग) चिंग में विस्तृत खड़ी विस्तारिता दिखती रही है, जबकि सूडोड्राइनरिया कोरोनैन्स (वाल एक्स हुक) चिंग, माइक्रोसोरम पंकटैटम (लीनियस) कोपेल, नेफ्रोलेपिक कार्डीफोलिया (लीनियस) प्र०, लेपीसोरस अस्सूरियेन्सिस (रीगेल एट माक) चिंग, पालिपोडियास्ट्रम आर्ग्यूटम (वाल एक्स हुक) चिंग, पाइरोसिया मैनाई (गीज) चिंग, सिलैजीनेला इन्वाल्वेन्स (स्वू) स्प्रिंग तथा टिनोप्टेरिस सबफैल्काटा ब्लूम एक्स कुन्जे में पोषी पौधों पर सीमित खड़ी विस्तारिता दिखती है। विशेषज्ञ प्रजातियों, दो पर्णांगों को छोड़ कर ऊँचाई के अनुरूप विभिन्नता दर्शाती है। सामान्य प्रजातियों, विभिन्न ऊँचाइयों के क्षेत्रों में लगभग एक सी है।

सांकेतिक शब्द : खड़ी विस्तारिता, अधिपादप विभिन्नता, विशेषज्ञ प्रजातियों, सामान्य प्रजातियों, संरक्षण, सिक्किम।

3. Upper hill forests (1500-2700' m).
4. Rhododendron-Conifer zone forests (2700-4300m).
5. High altitude scrub and grassland (3600-4300m)

Epiphytic pteridophyte diversity also varies with the change of climatic conditions and altitudinal ranges. In

pteridophytes, most of the members of the families of Lycopodiaceae, Vittariaceae, Hymenophyllaceae, Davalliaceae, Aspleniaceae and Polypodiaceae are adapted to epiphytic mode of life and they contribute a significant proportion to the pteridophytic flora particularly in Sikkim Himalaya. Epiphytes comprise about 10% of the total vascular plants of the world² but in Darjeeling and Sikkim Himalaya³ it is about 20%.

Apart from traditional systematic classification, the epiphytic pteridophytes have been classified in several ways³⁻⁹, mainly on the basis of nature of habitats and their associations. According to the vertical height sections, host trees are divided into four major regions⁷⁻⁸, which are (I) foot region, (II) upper foot region, (III) branch region and (IV) tree top region. Epiphytic pteridophytes grow in different positions on the host trees. This pattern is known as vertical distribution⁷. In India, many workers studied the floristic composition of pteridophytes since the time of Beddome¹⁰. Many workers¹¹⁻¹⁶ also contributed to the understanding of ecology of epiphytes. Workers outside India^{2, 7, 8, 17-20} contributed on the vertical distribution and ecology of vascular epiphytes, but very little work²¹ has been done in India regarding the vertical distribution and diversity of epiphytic pteridophytes with special reference to Sikkim Himalayas. So, this study was undertaken with a view to determine:

- a. The composition, pattern of vertical distribution and richness of epiphytic pteridophytes of southern Sikkim.
- b. The specialist epiphytic species and generalist epiphytic species.
- c. The diversity of epiphytic pteridophytes in different altitudinal zones.
- d. Whether the vertical distribution and occurrence of epiphytic pteridophytes are related to the type of forest, altitude, environmental factors or other characteristics of trees that serve as host.
- e. The epiphytic species rich areas of southern Sikkim, which could be recommended for *in situ* conservation for preserving biodiversity.

Material and Methods

During field survey to southern Sikkim for about 4 years, (2002-2006) several places were visited.

Southern Sikkim is a large area comprising about 3500 sq km. The study area was divided into 6x6 km grid cells following Bolos and Romo's²² method. As forest allow greater establishment and success of epiphyte vegetation²³, 28 grid cells of well dense forests, including wildlife sanctuaries, were chosen for study. Trees were selected randomly within a grid cell where epiphytic growth was present. They were subjected to fulfill the following parameters.

1. They are in good health.
2. They could be climbed.
3. They did not have exfoliating bark, because this did not allow establishment of stable epiphytic communities.

Using simple climbing equipments, the epiphytic pteridophytes were sampled on the host trees and the following data gathered:

1. Height of the host trees.
2. Kind of epiphytic species.
3. Height section of the host, from which the epiphytic species were collected.

Frequency of the epiphytic pteridophytes was calculated by using transect method from 28 grid cells.

Following Johansson⁷, Cornelison and Steege⁸ the host trees were divided into different height sections. Collections were identified with the help of authenticated specimens at Central National Herbarium (CAL) and published literatures^{3, 10, 24-26}. The altitudinal classifications of vegetation given by Humboldt¹ and Champion and Seth²⁷ were followed. Species richness with different environmental and geographical factors was analyzed statistically.

Observations

Among the 28 grid cells of southern Sikkim studied for diversity and vertical distribution of epiphytic pteridophytes, different places having different frequency (f) of occurrences were noted. Frequencies of each epiphytic pteridophyte along the altitudinal zones are given in Table 1.

The low and middle hill forest zone (up to 1500 m) had only 20 epiphytic pteridophytic species distributed vertically in the first three regions of the host trees.

Table 1 - Frequency of occurrence of epiphytic pteridophytes in different altitudinal zones

Sl. No.	Sc. Name(s) of the plants	Frequency in Low and Middle hill forest zone (Upto 1500m) \pm SD	Frequency in upper hill forest zone (1500-2700m) \pm SD	Frequency in Rhododendron-Conifer zone (2700-3600m) \pm SD
1.	<i>Asplenium ensiforme</i> Wall. ex Hook. & Grev	-	43.1 \pm 2.6	61.8 \pm 3.4
2.	<i>Asplenium nidus</i> Linn.	40.6 \pm 1.89	42.2 \pm 1.2	-
3.	<i>Asplenium nessu</i> Christ	-	28.4 \pm 3.3	14.2 \pm 0.72
4.	<i>Arthromeris himalayensis</i> (Hook.) Ching	-	31.3 \pm 2.9	67.5 \pm 7.1
5.	<i>Arthromeris wallichiana</i> (Spreng.) Ching	74.75 \pm 9.4	52.6 \pm 1.5	37.1 \pm 1.9
6.	<i>Araostegia pulchra</i> (D.Don) Copel	-	41.5 \pm 1.3	-
7.	<i>Araostegia beddomei</i> (Hope) Ching	-	15.0 \pm 8.9	-
8.	<i>Ctenopteris subfalcata</i> Blume ex Kunze	-	-	22.5 \pm 0.9
9.	<i>Crypsinus griffithiana</i> (Hook.) Copel.	-	40.9 \pm 2.7	-
10.	<i>Drynaria quercifolia</i> (L.) J.Sm.	43.3 \pm 2.3	12.2 \pm 0.7	-
11.	<i>Hymenophyllum simonsianum</i> Hook.	-	-	14.2 \pm 1.7
12.	<i>Lycopodium phlegmaria</i> Linn.	-	20.3 \pm 1.2	-
13.	<i>Loxogramme involuta</i> (D.Don) Presl	28.3 \pm 1.21	11.6 \pm 0.5	-
14.	<i>Loxogramme carinata</i> Price	-	11.1 \pm 0.7	-
15.	<i>Lepisorus clathratus</i> (Clarke) Ching	-	17.3 \pm 1.1	-
16.	<i>Lepisorus mehrae</i> Fras.-Jenk.	-	19.2 \pm 2.1	28.5 \pm 2.0
17.	<i>Lepisorus loriformis</i> (Wall. ex Mett.) Ching	-	20.0 \pm 1.9	38.7 \pm 2.9
18.	<i>Lepisorus loriformis</i> var. <i>steniste</i> sensu Bedd.	-	-	33.3 \pm 1.8
19.	<i>Lepisorus morrisonensis</i> (Hayata) H. Ito	12.5 \pm 6.7	22.7 \pm 1.2	-
20.	<i>Lepisorus nudus</i> (Hook.) Ching	50.2 \pm 4.0	38.0 \pm 3.3	13.3 \pm 1.4
21.	<i>Lepisorus thunbergianus</i> (Kaulf.) Ching	40.0 \pm 2.0	30.2 \pm 1.9	9.5 \pm 0.6
22.	<i>Lepisorus ussuriensis</i> (Regel et Maack) Ching	-	14.2 \pm 0.3	21.3 \pm 1.7
23.	<i>Lepisorus bicolor</i> Ching	-	12.5 \pm 1.3	-
24.	<i>Lepisorus kuchenensis</i> (Wu) Ching	-	20.3 \pm 2.0	-
25.	<i>Lepisorus sesquipetalis</i> (J.Sm.) Fras.-Jenk.	-	28.5 \pm 2.1	23.7 \pm 2.3
26.	<i>Leucostegia immersa</i> Wall. ex Presl	-	31.4 \pm 3.0	16.1 \pm 1.2
27.	<i>Lepidogrammitis rostrata</i> (Bedd.) Ching & SK Wu in Wu	-	21.6 \pm 3.2	-
28.	<i>Microsorium membranaceum</i> (D.Don) Ching	34.7 \pm 3.3	20.8 \pm 2.0	-
29.	<i>Microsorium punctatum</i> (L.) Copel.	47.7 \pm 3.3	10.0 \pm 0.5	-

30.	<i>Mecodiutn exsertum</i> (Wall. ex Hook) Copel.	-	26.6±1.3	33.9±2.7
31.	<i>Mecodium badium</i> (Hook. et Grev.) Copel.	-	-	23.7±1.9
32.	<i>Nephrolepis cordifolia</i> (L) Pr	73.1±8.0	51.2±5.2	16.4±1.1
33.	<i>Neocheiropteris normalis</i> (D.Don) Taga.	-	12.5±0.9	-
34.	<i>Neocheiropteris zipelli</i> (Bl.) Bosnian	-	28.7±2.1	-
35.	<i>Oleandra neriformis</i> Cav.	-	11.1±0.6	-
36.	<i>Oleandra wallichii</i> (Hook) Pr.	12.5±1.0	55.4±2.7	42.2±2.4
37.	<i>Pyrrosia flocculosa</i> (D Don) Ching	-	25.7±1.3	-
38.	<i>Pyrrosia lanceolata</i> (L.) Farwell	64.0±6.2	24.3±2.3	-
39.	<i>Pyrrosia manu</i> (Gies) Ching	28.5±2.1	14.2±1.0	-
40.	<i>Pyrrosia nuda</i> Ching	40.0±3.1	10.0±0.3	-
41.	<i>Pyrrosia pannosa</i> (Mett. ex Kuhn) Ching	22.2±2.1	16.5±1.7	-
42.	<i>Polypodiodes amoenum</i> (Wall. ex Mett.) Ching	-	32.3±2.8	41.4±3.2
43.	<i>Polypodiodes lachnopus</i> (Wall. ex Hook.) Ching	-	22.5±2.2	-
44.	<i>Polypodiodes microrrhizoma</i> (Clarke ex Baker) Ching	20.0±1.3	27.0±2.8	23.75±1.7
45.	<i>Polypodiastrium argutum</i> (Wall. ex Hook.) Ching	-	22.7±2.6	14.2±1.0
46.	<i>Phymatopteris ebenipes</i> (Hook.) Pic. Ser.	-	12.5±1.0	-
47.	<i>Phymatosoritis cuspidatus</i> (D.Don) Pic. Ser.	20.0±1.7	47.5±2.4	14.2±0.5
48.	<i>Pseudodrynaria coronans</i> (Wall. ex Hook) Ching	40.7±4.1	22.5±0.7	-
49.	<i>Selaginella involvens</i> (Sw.) Spring	-	20.0±1.3	15.4±1.5
50.	<i>Vittaria elongata</i> Sw.	11.8±1.2	11.1±1.5	-
51.	<i>Vittaria himalayensis</i> Ching	-	30.0±2.8	-
52.	<i>Vittaria linearifolia</i> Ching	-	11.1±0.7	-
53.	<i>Vittaria ophiopogonoides</i> Ching	16.6±2.1	23.7±2.0	-
54.	<i>Vittaria taeniophylla</i> Copel	-	33.3±4.1	34.2±4.3

The tree top region had a single epiphytic pteridophyte. The species found in low and middle hill forest zone develop marked contrivances for storing their available water forming nest like or bracket like structures and thick cuticularised leaves [*Pseudodrynaria coronans* (Wall. ex Hook) Ching, *Drynaria quercifolia* (L.) J. Sm., [*Asplenium nidus* Linn], by inrolling of leaves to prevent desiccation [*Lepisorus nudus* (Hook) Ching] and by the development of thick woollen hair on the leaf surface for preventing excessive transpiration [*Pyrrosia pannosa* (Mett. ex Kuhn) Ching]. Succulent epiphytes like, *Microsorium punctatum* (L.) Copel, *Pyrrosia* sp., *Vittaria* sp. and *Asplenium nidus* Linn. have physiologi-

cal adaptation by operating CAM (Crassulacean acid metabolism)²⁸⁻²⁹.

The host trees found in the low and middle hill forest zone were *Shorea robusta* Gaertn.f., *Schima wallichii* Choisy, *Bauhinia purpurea* Linn. *Cedrela toona* Roxb.ex Rottl., *Pterospermum tetragonum* DC., *Salmalia malabarica* (DC.) Schott and Endl., *Sterculia vittosa* Roxb., *Terminalia myriocarpa* Van Heurck and Muell. Arg., *Albizia lebbek* (L.) Benth., *Tectona grandis* Linn. The average height of the host trees was 30m. Among the 21 epiphytic pteridophytes, *Pseudodrynaria coronans* (Wall ex Hook) Ching *Microsorium punctatum* (L) Copel

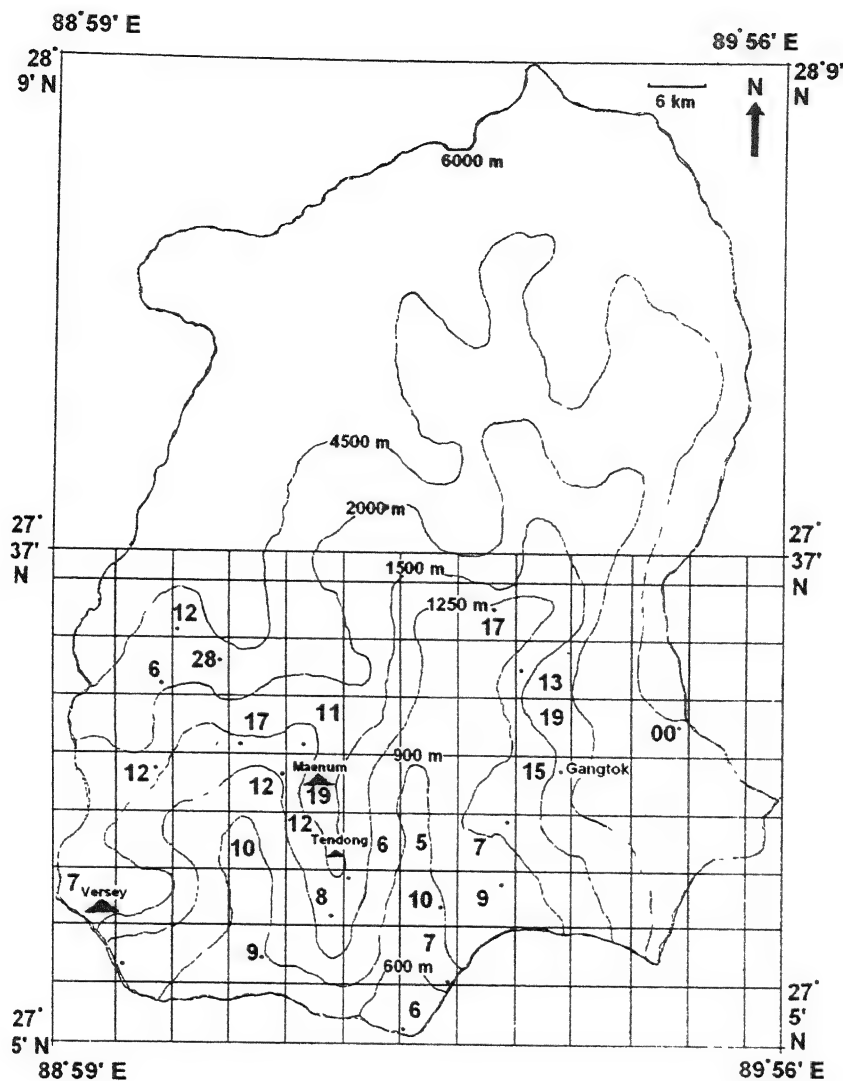


Fig. 1 - Map of Sikkim showing distribution of number of epiphytic pteridophytic species in the grid cells along different altitudes.

and *Lycopodium sguarrosus* had restricted distributions on trees, while other species were distributed in two or three vertical regions (Table 2).

Upper hill forests consisted of host trees such as *Quercus lamellosa* Sm., *Q. pachyphylla* Kunz, *Q. lineata* Blume, *Magnolia campbelli* Hook. f. and Thoms., *Betula alnoides* Buch.-Ham., *Acer calmpbelli* Hook.f. and Thoms, *Rhododendron griffithianum* Wight, with an average height of 24 m. This region was the ideal habitat of epiphytic pteridophytes harbouring diverse types of pteridophytes. Temperature of this region ranged from 5°C-18°C, almost throughout the year, except in winter season; high humidity and heavy rainfall, which measure about 282 cm annually (average of 28 grid cells)

prevailed during monsoon, provided ideal growth conditions for epiphytic pteridophytes. Fifty species of epiphytic pteridophytes were recorded from this zone (Table 2). Among these, only *Oleandra wallichii* (Hook.) Pr. was found to be distributed widely in all the four vertical regions of the host trees while, *Nephrolepis cordifolia* (L.) Pr., *Lepisorus ussuriensis* (Regel et Maack) Ching, *Polypodiastrium argutum* (Wall. ex Hook.) Ching, *Pseudodrynaria coronans* (Wall. ex Hook.) Ching, *Pyrrosia manii* (Gies.) Ching, *Selaginella involvens* (Sw.) Spring and *Ctenopteris subfalcata* Blume ex Kunze were found to have restricted vertical distributions in either the foot region or in the upper foot region. *Polypodioides lachnopus* (Wall. ex Hook.) Ching was found to have wide vertical distributions except in the

tree top region. *Lepisorus mehrae* and *Lepisorus sesquipedalis* (J.Sm.) Fras.-Jenk. were found in tree top region in addition to *Oleandra wallichii* (Hook.) Pr.

Asplenium ensiforme Wall. ex Hook., *Lepisorus loriformis* (Wall. ex Mett.) Ching, *Lepisorus loriformis* var. *steniste* sensu Bedd., *Crypsinus griffithiana* (Hook.) Copel., *Vittaria ophiopogonoides* Ching, *V linearifolia* Ching, *V. taeniophylla* Copel., *V. himalayensis* Ching and *V. elongata* Sw. were found to survive in the months of November and December with fertile leaves. They showed some adaptive features like thick fleshy leaves with highly cuticularised epidermal layer or narrow lanceolate leaves, which helped them to withstand the adverse conditions. Out of the total 50 species occurring in the upper hill forest zone, 45 were found in the upper foot region and 33 in the branch region of the host trees (Table: 3).

Rhododendron-Conifer zone forests of Sikkim are situated between 2700-3600 m altitudes. Temperature of this zone is almost below 10°C throughout the year, except in winter months, when it is below freezing temperature. This zone consisted of host trees such as *Quercus lineata* Blume, *Acer campbelli* Hook.f. and Thoms, *Betula utilis* D.Don, *Magnolia campbelli* Hook.f. and Thoms, *Rhododendron arboreum* Sin., *R. campanulatum* Don, *Tsuga brunoniana* Carr., *Abies densa* Griff., *Cryptomeria japonica* D.Don, *Pinus* spp., *Rhododendron setosum* D.Don. The average height of the host trees of this region was 18 m. This zone harbours only 24 epiphytic pteridophytes. Out of them, *Oleandra wallichii* (Hook.) Pr., *Lepisorus sesquipedalis* (J.Sm.) Fras.-Jenk. and *Arthromeris wallichiana* (Spreng.) Ching, had wide vertical distributions, except at the tree top region. *Lepidogrammitis rostrata* (Bedd.) Ching and Sk Wu in Wu. *Ctenopteris subfalcata* Blume ex Kunze, *Hymenophyllum simonsianum* Hook, and *Selaginella involvens* (Sw.) Spring, were found to have very restricted vertical distributions. It was observed that in the Rhododendron-Conifer zone, *Rhododendron* spp. and some conifers did not harbour much epiphytic pteridophytes. Only two pteridophytic species [*Arthromeris himalayensis* (Hook.) Ching and *Lepisorus loriformis* (Wall. ex Mett.) Ching], were found to grow on trunks of *Rhododendron* spp. having smooth type of bark. *Quercus lineata* Blume common in this region acted as good host for epiphytes due to the corrosive nature of the bark.

Table 2 shows that the upper foot region of each of the three-altitudinal zones have the maximum diver-

sity of pteridophytes. Species richness (a simple count of the number of species), was far greater in the upper foot and branch regions than the lower foot and tree top regions. In the low and middle hill forest zones tree top region showed poorest occurrence of epiphytic pteridophytes. The highest diversity of epiphytic pteridophytes was in the upper foot regions of the host trees in the upper hill forest area.

Analysis of correlation between species richness with different environmental and geographical factors showed that correlation value with rainfall ($r^2 = 0.3278$), relative humidity ($r^2 = 0.2825$), annual minimum and maximum temperatures ($r^2 = 0.0075$ to 0.1475) and altitude ($r^2 = 0.004$) varies considerably.

Discussion

Epiphytic pteridophytes are represented in southern Sikkim by 53 species and one variety. Polypodiaceae is the largest and dominant family of this area represented by 31 species and one variety, followed by Vittariaceae with five species and Aspleniaceae, Hymenophyllaceae and Davalliaceae each represented by three species. Other epiphytic families are represented by one or two species.

All the epiphytic members are distributed in the different altitudinal regions (Fig. 1). The diversity of epiphytic pteridophytes increases with the altitude up to a certain level. From 2700 m upward, diversity of epiphytic pteridophytes decreases. Actually, in low and middle hill forest zone the prevailing adverse conditions like high temperature (average 18°C-35°C throughout the year, except in winter), low humidity etc. hinder the growth of epiphytic pteridophytes and decrease the chances of epiphytic establishment and growth. Relative humidity (RH) is a vital component for the establishment of epiphytic habit. Incidence of poor epiphytic habit in low and middle hill forest zone and in coniferous forest zone results from the prevalence of deciduous type of trees and reduced leaved coniferous type of trees respectively. On the contrary, rich epiphytic habit, which is present in the upper hill forest zone, results due to the presence of evergreen broad-leaved forest trees. Statistical analysis also support this view. From statistical data analysis it was found that, epiphytic species richness was highly correlated with average annual rainfall ($r^2 = 0.3278$) and average relative humidity ($r^2 = 0.2825$). Weaker correlations were found in case of annual average temperature ($r^2 = 0.0075$ to

Table 2 - Epiphytic species distribution in different regions of the host trees in relation to the different altitudinal forest types.

Sl. No.	Epiphytic pteridophytes	Low & middle hill forest zone (upto 1500m)				Upper hill forest zone (1500-2700 m)				Rhododendron conifer zone (2700-3600)			
		R I	R II	R III	R IV	R I	R II	R III	R IV	R I	R II	R III	R IV
1.	<i>Asplenium ensiforme</i>	-	-	-	-	-	+	+	-	-	+	+	-
2.	<i>Asplenium nidus</i>	-	+	+	-	-	+	+	-	-	-	-	-
3.	<i>Asplenium nessi</i>	-	-	-	-	-	+	-	-	-	+	-	-
4.	<i>Arthromeris humalayans</i>	-	-	-	-	-	-	-	-	-	+	-	-
5.	<i>Arthromeris wallichiana</i>	+	+	-	-	-	+	+	-	+	+	-	-
6.	<i>Araiostegia pulchra</i>	-	-	-	-	+	+	-	-	-	-	-	-
7.	<i>Araiostegia beddomei</i>	-	-	-	-	+	-	-	-	-	-	-	-
8.	<i>Ctenopteris subfalcata</i>	-	-	-	-	-	-	-	-	+	+	-	-
9.	<i>Crypsinus griffithiana</i>	-	-	-	-	-	+	+	-	-	-	-	-
10.	<i>Drynaria quercifolia</i>	-	+	+	-	-	+	+	-	-	-	-	-
11.	<i>Hymenophyllum simonsianum</i>	-	-	-	-	-	-	-	-	-	+	+	-
12.	<i>Lycopodium phlegmaria</i>	-	-	-	-	-	+	-	-	-	-	-	-
13.	<i>Loxogramme involuta</i>	-	+	+	-	-	+	+	-	-	-	-	-
14.	<i>Loxogramme carinata</i>	-	-	-	-	-	+	+	-	-	-	-	-
15.	<i>Lepisorus clathratus</i>	-	-	-	-	-	+	-	-	-	-	-	-
16.	<i>Lepisorus mehrae</i>	-	-	-	-	-	-	+	+	-	-	+	-
17.	<i>Lepisorus loriformis</i>	-	-	-	-	-	+	+	-	-	+	-	-
18.	<i>Lepisorus loriformis</i> var. <i>steniste</i>	-	-	-	-	-	-	-	-	-	+	-	-
19.	<i>Lepisorus morrisonensis</i>	-	+	-	-	-	+	-	-	-	-	-	-
20.	<i>Lepisorus nudus</i>	+	+	+	-	+	+	+	-	+	+	-	-
21.	<i>Lepisorus thunbergianus</i>	-	+	+	-	+	+	+	-	+	+	-	-
22.	<i>Lepisorus kuchenensis</i>	-	-	-	-	-	+	+	-	-	-	-	-
23.	<i>Lepisorus sesquipetalis</i>	-	-	-	-	-	+	-	-	-	+	+	-
24.	<i>Lepisorus ussuriensis</i>	-	-	-	-	-	+	-	-	-	+	+	-
25.	<i>Lepisorus bicolor</i>	-	-	-	-	-	+	-	-	-	-	-	-
26.	<i>Leucostegia immersa</i>	-	-	-	-	+	+	-	-	-	+	+	-
27.	<i>Lepidogrammitis rostrata</i>				-	+	+	-	-	-	-	-	
28.	<i>Microsorium membranaceum</i>	-	+	+	-	-	+	+	-	-	-	-	-
29.	<i>Microsorium punctatum</i>	-	-	+	-	-	+	+	-	-	-	-	-

30. <i>Mecodium exsertum</i>	-	-	-	-	+	+	-	-	+	+	-	-
31. <i>Mecodium badium</i>	-	-	-	-	-	-	-	-	+	+	-	-
32. <i>Nephrolepis cordifolia</i>	+	+	-	-	+	-	-	-	+	-	-	-
33. <i>Neocheiropteris normalis</i>	-	-	-	-	-	+	+	-	-	-	-	-
34. <i>Neocheiropteris zipelli</i>	-	-	-	-	-	+	+	-	-	-	-	-
35. <i>Oleandra neriformis</i>	-	-	-	-	-	+	-	-	-	-	-	-
36. <i>Oleandra wallichii</i>	+	+	+	-	+	+	+	+	-	+	+	+
37. <i>Pyrrosia flocculosa</i>	-	-	-	-	-	+	+	-	-	-	-	-
38. <i>Pyrrosia lanceolata</i>	-	+	+	-	-	+	+	-	-	-	-	-
39. <i>Pyrrosia manu</i>	-	+	+	-	-	+	-	-	-	-	-	-
40. <i>Pyrrosia pannosa</i>	-	+	+	-	-	+	+	-	-	-	-	-
41. <i>Pyrrosia nuda</i>	-	-	-	-	-	+	+	-	-	+	-	-
42. <i>Polypodiodes amoenum</i>	-	-	-	-	-	+	+	-	-	+	-	-
43. <i>Polypodiodes lachnopus</i>	-	+	+	-	-	+	+	-	-	+	+	-
44. <i>Polypodiastrium argutum</i>	-	-	-	-	-	+	+	-	-	-	-	-
45. <i>P. microrrhizoma</i>	-	-	-	-	-	+	+	-	-	+	+	-
46. <i>Phymatopteris ebenipes</i>				-	+	+	-	-	-	-	-	-
47. <i>Phymatosorus cuspidatus</i>	-	+	-	-	-	+	-	-	-	+	-	-
48. <i>Pseudodrynaria coronans</i>	-	+	-	-	-	+	+	-	-	-	-	-
49. <i>Selaginella involvew</i>		-	-	-	+	-	-	-	+	-	-	-
50. <i>Vittaria elongata</i>	-	+	+	-	-	+	+	-	-	-	-	-
51. <i>Vittaria himalayensis</i>	-			-	+	+	-	-	-	-	-	-
52. <i>Vittaria linearifolia</i>	-	-	-	-	-	+	+	-	-	-	-	-
53. <i>Vittaria ophiopogonoides</i>	-	+	+	-	-	+	+	-	-	-	-	-
54. <i>Vittaria taeniophylla</i>	-	-	-	-	-	+	+	-	-	+	+	-

Table 3 - Epiphytic species-richness at different regions of the host plant in relation to altitudinal forest types.

Forest types along the altitudes	Height section of the host tree				Total epiphytic species
	Foot region (I)	Upper foot region (II)	Branch region (III)	Tree top region (IV)	
Low and middle hill forest (upto 1 500 m) zone	04	18	14	00	20
Upper hill forest zone (1500-2700m)	10	45	33	02	50
Rhododendron-conifer forest zone (2700-3600m)	09	23	10	01	24

NB: The same epiphyte may develop in more than one region.

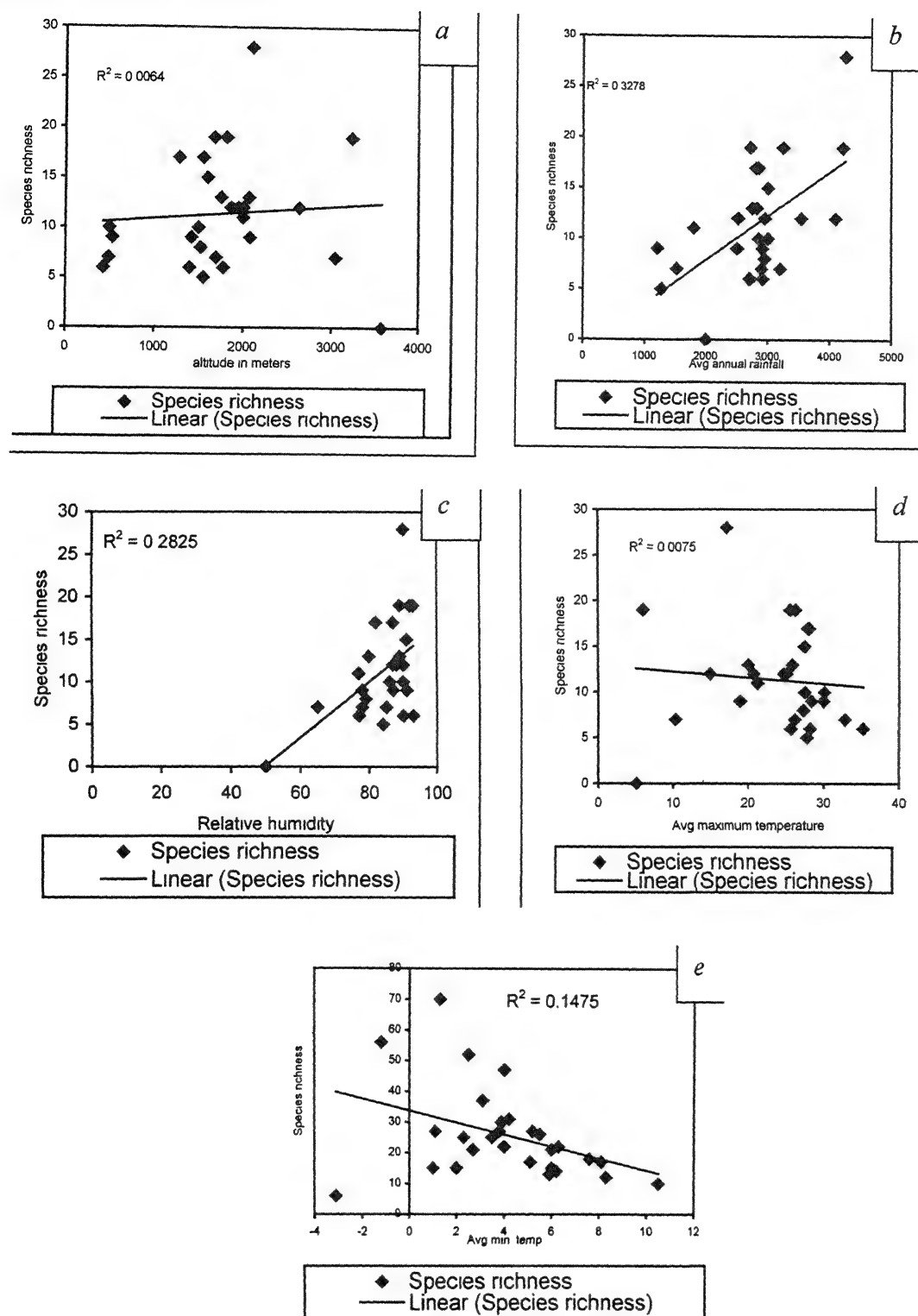


Fig. 2 - a. Variation of epiphytic species richness along the altitudinal gradient in South Sikkim. b. Change of epiphytic species richness with average annual rainfall in South Sikkim. c. Change of epiphytic species richness with relative humidity. d. Effect of average maximum temperature on epiphytic species richness of different places of South Sikkim. e. Effect of average minimum temperature on epiphytic species richness of different places of South Sikkim.

$r^2 = 0.1475$) and altitudinal variations ($r^2 = 0.004$). Only a few species like *Pseudodrynaria coronans* (Wall, ex Hook.) Ching, *Drynaria quercifolia* (L.) J.Sm., *Lepisorus nudus* (Hook.) Ching and *Pyrrosia pannosa* (Mett. ex Kuhn.) Ching and some other species can withstand these conditions by developing the following adaptations.

- i. Nest-like or bracket-like structure.
- ii. Thick cuticularised leaves.
- iii. Presence of thick woolen hair etc.

Upper hill forest zone shows the maximum diversity of epiphytic pteridophytes. About 91% of the total number of epiphytic pteridophytes is found in this zone. This zone provides the most congenial conditions of temperature (5°C-18°C) and humidity for growth of epiphytic pteridophytes. Rhododendron-Conifer zone occupies the intermediate position between Low and Middle hill and Upper hill forest zones regarding epiphytic pteridophyte diversity.

From the point of view of preference of altitudinal zones for frequency of occurrences, *Arthromeris wallichiana* (Spreng.) Ching, *Lepisorus nudus* (Hook.) Ching, *L. thunbergianus* (Kaulf.) Ching and *Nephrolepis cordifolia* (L.) Pr. show decreasing tendency in the number with the increase of altitude, whereas in *Lepisorus sesquipedalis* (J.Sm.) Fras.-Jenk., it is reverse. Some species like *Oleandra wallichii*, *Plypodioides lachnopus* and *Phymatosorus cuspidatus* (D.Don) Pic. Ser. had maximum frequency in the mid altitude (1500-2700 m). On the other hand, species like *Ctenopteris subfalcata* Blume ex Kunze, *Hymenophyllum simonsianum* Hook., *Lepisorus loriformis* var. *steniste* sensu Bedd., *Mecodium badium* (Hook. et Grev.) Copel, are restricted to higher altitudes (2700-3600 m) with low frequency of occurrence. Sixteen species of epiphytic pteridophytes prefer only the upper hill (1500-2700 m) forest zone; they are neither found in low and middle hill forest zones, nor in the Rhododendron-Conifer zone (Table 2).

The vertical distribution of epiphytic pteridophytes is mostly confined to one or two regions on the host trees for the majority of species, except a few species, which are widely distributed throughout the three vertical regions. Depending on their wide or restricted distributions, epiphytic pteridophytes are divided into two categories: - i. Generalist species (having wide vertical distribution) and ii. Specialist species (having restricted

vertical distribution). Regarding richness of epiphytic pteridophytic species on different vertical regions, upper foot region bears the maximum number of epiphytic pteridophytes. Upper foot region starts from 6 m and ranges up to 20 m. So, this region provides suitable microhabitat for growth of epiphytic pteridophytes due to the presence of high humidity, ideal range of temperatures (5°C-18°C), low wind velocity and being out of reach of grazing animals. On the other hand, the tree top region showing the lowest diversity of epiphytic pteridophytes is probably due to adverse climatic conditions like low humidity, low temperature and high wind velocity.

Epiphytic species richness is highest in the Upper hill forest zone and prevalence of epiphytes is maximum in the upper foot region of the host trees (Table 3).

As the upper hill forest areas of Sikkim harbour the highest epiphytic pteridophyte diversity this region may be considered as most suitable for the recommendation of *in situ* conservation for preserving the epiphytic pteridophyte biodiversity.

Acknowledgements

Authors are grateful to the Council of Scientific and Industrial Research (CSIR), New Delhi, India for financial assistance and to the Meteorological Department, Govt. of Sikkim for providing necessary help.

References

1. Humboldt BA. Physical geography of Sikkim Himalaya. *Hook J Bot* 1851; 3 : 21-31.
2. Hietz P. Diversity and Conservation of epiphytes in a changing environment. *Pure and Applied Chemistry* 1998; 70(11) : 1-7.
3. Mehra PN, Bir SS. Pteridophytic flora of Darjeeling and Sikkim Himalayas. *Res Bull Punjab Univ* 1964; 15(1&2): 69-182.
4. Sands WW. Epiphytes on cultivated tree. *Malaya Agric J* 1926; 14 : 13-17.
5. Holttum RE. The ecology of tropical pteridophytes. In: Vaerdoon F, editor. *Manual of Pteridology* The Hague: Martinus Nijhoff 1938; 420-450
6. Dhir KK. Ferns of North-western Himalayas. *Bibliotheca Pteridologia* 1980; 1 : 1-58.
7. Johansson D. Ecology of vascular epiphytes in West African rain forest. *Acta Phytogeography Suec* 1974; 59 : 1-129.

8. Cornelison JHC, Steege H. Distribution and ecology of vascular epiphytes in low land forest of Guyana. *J Trop Ecol* 1989; **5** : 131-150.
9. Page CN. The diversity of ferns-An ecological perspectives, In: Dyer AF, editor. The experimental biology of ferns. London, NY, Sanfransisco: Academic Press, 1979. 9-56.
10. Beddome RH. Handbook to the Ferns of British India, Ceylon and Malaya peninsula. Calcutta Thacker Spink and Co 1883. 1-500.
11. Bir SS. Ecological classification and distribution of polypodiaceous ferns of the Himalayas. Appendix-I In: Satija CK, Bir SS. editors Aspects of plant sciences VIII. New Delhi : Today and Tomorrow Printers and Publishers. 1985 104-110
12. Bir SS, Satija CK, Bir P. Studies on the India Fern Ecology, Distribution and Phytogeography of Polypodiaceae. In : Bir SS editor Aspects of plant sciences New Delhi : Today and Tomorrow Printers and Publishers, 1983. 1-24.
13. Manickam VS, Ninan CA. Ecological studies on the Fern Flora of Palni Hills (South India). New Delhi . Today and Tomorrow Printers and Publishers, 1984.
14. Mehra PN. Ferns of Mussorie. Lahore: Panjab Univ. Publ, 1939.
15. King LJ. Weed of the world biology and control. New Delhi: Wiley Eastern Private Ltd , 1966.
16. Madison M. Vascular epiphytes, their systematic occurrence and salient features *Selbyana* 1977, **2** : 1-13.
17. Ingram SW. The abundance, vegetative composition and distribution of epiphytes in Costa Rican lower montane rain forest. M Sc thesis. University of California Santa Barbara USA 1999.
18. Bussmann RW, Werner F, Schoaf A, Bogh A. Composition and distribution of vascular epiphyte flora of an Ecuadorian Montane Rain Forest. *Selbyana* 1992, **13** : 25-34.
19. Wolf JAD. Ecology of epiphytic communities in montane rain forests, PhD Thesis University of Amsterdam. 1993
20. Nieder J, Engward S, Klawn P, Barthlott W. Spatial distribution of vascular epiphytes (including hemiepiphytes) in a lowland Amazonian rain forest (Surumoni crane plot) in southern Venezuela. *Biotropica* 2000; **32**(3) : 21-29.
21. Khullar SP. Frequency of distribution of epiphytic ferns in Himalayas. In: Verma SC, editor. Contemporary trends in plant sciences Ludhiana and New Delhi : Kalyani Publishers, 1982. 202-207.
22. Bolos O, Romo AM. Atlas corologic de la flora vascular dels Països Catalans. Vol. II Bercelona : Institute d' Estudis Catalans, 1991.
23. Barthlott WV, Neuburg S, Nieder J, Engwald S. Diversity and abundance of vascular epiphytes: a comparison of secondary vegetation and primary montane rain forest in the Venezuelan Andes *Plant Ecology* 2001; **152** : 145-156.
24. Khullar SP. An illustrated Fern Flora of West Himalaya, Vol. I. Dehra Dun : International book distributors, 1994.
25. Khullar SP. An illustrated Fern Flora of West Himalaya, Vol. II, Dehra Dun : International book distributors, 2000
26. Fraser-Jenkins CR. New species syndrome in Indian Pteridophytes and the ferns of Nepal. Dehra Dun : International book distributors, 1997.
27. Champion H, Seth SK. A revised survey of forests of India, New Delhi. Govt of India Publ Division, 1968
28. Ting I P. Crassulacean Acid Metabolism. *Ann Rev Plant Physiol* 1985; **36** : 595-622 .
29. Irudayaraj V, Jeyanath, H. Diurnal Fluctuations of acid and starch in a succulent epiphytic medicinal fern *Microsorium punctatum* (L.) Copel (Polypodiaceae. Pteridophyta). *Indian Fern J* 1999; **16** : 44-47.

***In-situ* and *ex-situ* evaluation of seaweed liquid fertilizers on seed germination, early growth and chlorophyll content of *Trigonella foenum graecum* Linn.**

VAIBHAV A. MANTRI* and B.B. CHAUGULE

Department of Botany, University of Pune, Ganeshkhind, Pune-411007, India.

*Present Address : Marine Biotechnology and Ecology Discipline, Central Salt and Marine Chemicals Research Institute (CSIR), Gijubhai Badheka Marg, Bhavnagar-364002, India.

*e-mail : vaibhav@csmcri.org

Received September 11, 2006, Revised August 29, 2007, Accepted September 24, 2007

Abstract

The effects of seaweed liquid fertilizers (SLFs) prepared from *Sargassum tenerrimum* J. Ag., *Hypnea valentiae* Turn and *Soheria robusta* (Grev) Kylin on seed germination, early growth and chlorophyll content of *Trigonella foenum graecum* Linn. were evaluated *in-situ* and *ex-situ*. Among different seaweed concentrations tested *in-situ* viz 10%, 30%, 50%, 70% and 100%, 10% extract of all the seaweeds studied was found to promote germination, hypocotyl and root elongation. SLFs prepared from dried seaweeds were more effective than fresh seaweeds at all the concentrations tested. Under *ex-situ* conditions 10% *Hypnea valentiae* extract showed one to two fold increase in root length, shoot length and leaf area and two fold increase in total chlorophyll content.

Key words : seaweed extract, seed germination, hypocotyl elongation, root elongation.

Introduction

India, with more than 8000 km coastal stretch, harbors about 900 different seaweeds with plentiful biomass. These have hardly had been utilized by industries except for hydrocolloid production. Recently, a few seaweed utilizing industries in southern India are turning towards the large scale production of Seaweed Liquid Fertilizers (SLFs)¹. While, historical records show that the use of seaweeds in agriculture is a very old and routine practice widely followed especially in Europe², its use has yet to be established in Indian agriculture. According to Thivy³, in India there is a causal use of seaweeds for agricultural purpose in places like Ratnagiri, Goa, Karwar and Tirunellveli. In India,

सारांश

विभिन्न समुद्री शैवालो सरगेसम टेनेरीमम जे०एजी०, हिप्निया व्हेलन्शी टर्न० एव सोलेरिया रोबस्टा (ग्रीव) काईलिन, से बनाए गये द्रव्य खाद (एस०एल०एफ०) का प्रभाव ट्रायगोनेला फोईनम ग्रासियम लिन के बीज अकुरण, आरम्भिक वृद्धि एव हरित द्रव्य मात्रा को अन्त अवस्थिति एव बाह्य अवस्थिति में मूल्यांकित किया गया। सभी समुद्री शैवालो के विभिन्न सान्द्रता के अर्क (जैसे कि 10%, 30%, 50%, 70% एव 100%) में से 10% सान्द्रता का अर्क बीज अकुरण वृद्धि कारक एव हाइपोकोटाईल एव मूल की लंबाई का वर्धक सिद्ध हुआ। सभी सान्द्रताओं में, सुखाये गये शैवालो का अर्क, ताजे शैवालो के अर्क से बेहतर पाया गया। बाह्य अवस्थिति में 10% हिप्निया व्हेलन्शी के अर्क के कारण मूल एव तने की लंबाई एव पत्ते का क्षेत्रफल एक से दो गुना हो गया तथा हरित द्रव्य की मात्रा भी दो गुनी हो गयी।

सांकेतिक शब्द : समुद्री शैवालीय अर्क, बीजाकुरण, हाइपोकोटाईल का लम्बा होना, मूल का लम्बा होना।

'ethanophycological' use of seaweeds as manure has also been documented very recently for coconut, mango, cashew-nut and jackfruit orchards of Malvan⁴⁻⁵. Apart from agricultural crop plants, seaweed extracts have been tested positive for vegetative growth in ornamental foliage and horticultural plants and enhancing growth of prokaryotic organisms⁶⁻⁹. The growth promoting activity of seaweed extracts is mainly due to presence of plant hormone like substances¹⁰⁻¹³, high levels of potassium¹⁴, vitamins¹⁵ and micro¹⁶⁻¹⁷ and macro¹⁷⁻¹⁹ nutrients. Such substances become readily available to crop plants when applied directly to the soil or used as foliar sprays. Seaweed liquid fertilizers also help plants in withstanding different stress conditions and infec-

tions²⁰⁻²³. The present study was aimed at evaluating the effects of seaweed liquid fertilizers (SLFs) prepared from *Sargassum tenerrimum* J. Ag., *Hypnea valentiae* Turn. and *Solieria robusta* (Grev.) Kylin on seed germination, early growth and chlorophyll content of *Trigonella foenum graecum* Linn. under *in-situ* and *ex-situ* conditions.

Material and Methods

The seaweeds were collected from Malvan coast (73.27 E and 16.03 N) in November, 1998. Selection of test seaweeds was made after interviewing farmers of Malvan, who have been using them traditionally, and detailed enquiry showed these as the preferred choice

amongst the other available seaweeds. The seaweeds were hand picked and washed thoroughly with seawater in the field itself to remove all the unwanted impurities like epiphytes and adhering sand particles and then brought immediately to the working laboratory and washed with tap water. Half the portion from the collection was directly used for extraction of liquid fertilizer while half was shade dried before extraction. Separate fertilizer extracts were prepared from dry and fresh seaweeds following the method described by Bhosle *et al*²⁴. 1000 gm of each fresh and dried algae were cut in to small pieces homogenized in 1000 ml distilled water in an electric blender and filtered. The resultant pull out is considered as the full strength extract (100%) and used in the further experiments. Different concen-

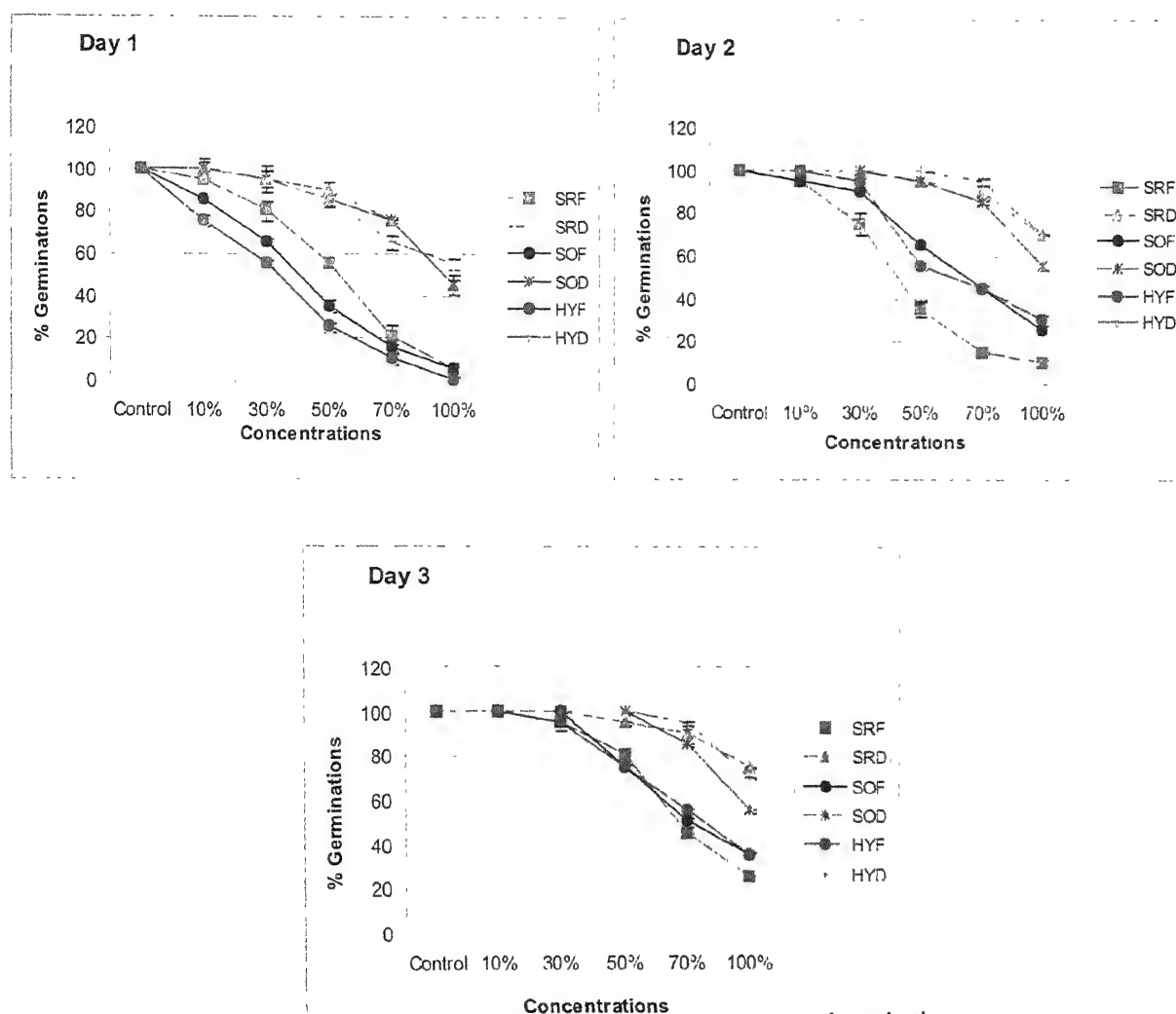


Fig. 1 - Effect of different seaweeds extracts on percentage seed germination.

*SRF : *Sargassum* Fresh extract; SRD: *Sargassum* Dry extract; SOF: *Solieria* Fresh extract; SOD: *Solieria* Dry extract, HYF: *Hypnea* Fresh extract & HYD: *Hypnea* Dry extract.

Table 1 - Effect of different seaweed extracts on hypocotyle and root elongation of *Trigonella foenum graecum*

Seaweed	Sargassum				Solieria				Hypnea			
	Hypocotyle elongation		Root elongation		Hypocotyle elongation		Root elongation		Hypocotyle elongation		Root elongation	
Concentrations	Fresh	Dry	Fresh	Dry	Fresh	Dry	Fresh	Dry	Fresh	Dry	Fresh	Dry
Control	2.47 (± 0.25)	3.90 (± 0.11)	1.44 (± 0.01)	3.22 (± 0.12)	2.47 (± 0.25)	3.90 (± 0.72)	1.44 (± 0.25)	3.22 (± 0.15)	2.47 (± 0.16)	3.90 (± 0.33)	1.44 (± 0.40)	3.22 (± 0.24)
10 %	0.57 (± 0.17)	5.02 (± 0.71)	0.50 (± 0.05)	5.42 (± 0.20)	1.80 (± 0.08)	6.33 (± 0.45)	1.46 (± 0.70)	7.48 (± 1.12)	2.20 (± 0.05)	5.02 (± 0.12)	2.20 (± 0.63)	5.42 (± 0.19)
30 %	2.00 (± 0.01)	4.65 (± 0.25)	2.42 (± 0.18)	5.95 (± 1.05)	2.68 (± 0.89)	5.66 (± 0.03)	2.24 (± 0.86)	6.63 (± 0.01)	0 (± 0)	4.65 (± 1.20)	2.10 (± 0.05)	5.95 (± 2.07)
50 %	2.48 (± 0.18)	5.36 (± 1.72)	2.93 (± 0.43)	5.75 (± 1.89)	2.55 (± 1.10)	2.48 (± 0.43)	2.04 (± 0.07)	3.56 (± 1.56)	0 (± 0)	5.36 (± 2.14)	2.00 (± 1.67)	5.75 (± 1.92)
70 %	2.88 (± 1.64)	4.89 (± 1.93)	2.96 (± 1.20)	5.24 (± 2.04)	1.15 (± 1.02)	0 (± 0)	1.06 (± 1.08)	2.75 (± 1.08)	0 (± 0)	4.89 (± 1.46)	1.80 (± 1.21)	5.24 (± 2.00)
100 %	1.39 (± 1.12)	4.99 (± 1.28)	2.04 (± 1.07)	4.69 (± 1.87)	0 (± 0)	0 (± 0)	1.30 (± 1.01)	0.35 (± 1.07)	0 (± 0)	4.99 (± 2.07)	0 (± 0)	4.69 (± 1.85)

Table 2 - Effect of 10 % dry Hypnea extract on *Trigonella foenum graecum* on growth parameters

Parameters	SLF Treatment	
	Control	Treated
Fresh wt (g)	0.25 (± 1.01)	0.87 (± 1.27)
Dry wt (g)	0.04 (± 0.01)	0.13 (± 0.08)
Shot length (cm)	11.79 (± 4.21)	13.63 (± 5.89)
Root length (cm)	5.1 (± 1.47)	8.02 (± 2.06)
Leaf area (mm ²)	532 (± 68.92)	769 (± 84.06)
Chlorophyll a (mg/g Fresh wt)	0.10 (± 0.04)	0.09 (± 0)
Chlorophyll b (mg/g Fresh wt)	0.06 (± 0)	0.12 (± 0.03)
Total Chlorophyll (mg/g Fresh wt)	0.16 (± 0.02)	0.22 (± 0.05)

trations of the seaweed extracts viz. 10%, 30%, 50%, 70% and 100% were made using distilled water. All the tests were carried out in duplicate.

Healthy, uniform seeds of *Trigonella foenum graecum* were pre-soaked in distilled water (control) and extracts of respective concentrations for 24 h and then equispacially placed (n = 10) in glass Petri dishes of 4" diameter with Whatman filter paper No. 1 at the

base. Ten ml of different concentrations of the SLFs was then poured in the Petri dishes. Distilled water was taken as control. Seed germination % was recorded for three successive days. Root and hypocotyl elongation were measured after eight days. Lower (10%) concentration of dry *H. valentiae* extract, which showed encouraging results, was selected for further *ex-situ* studies in the field. *Trigonella foenum graecum* plants were grown in two different plots side by side in the

Botanical garden, Department of Botany, University of Pune. Plants were watered every day for 30 days. In one plot, plants were applied 10% of dry *H. valentiae* extract as foliar drench on alternate days. The other plot was maintained without any foliar drench (control). At the end of the experiment, when plants were at their vegetative growth stage, five random plants from each plot were sampled and study was made of fresh weight, dry weight, shoot length, root length, leaf area and chlorophyll.

Results and Discussion

In-situ evaluation of seed germination against varying concentrations of the seaweed extracts (Figure 1) showed 100 % seed germination in control on first day itself. Seeds treated with algal extracts showed maximum germination % only at lower concentrations. In case of dry extract of *Sargassum tenerrimum*, *Hypnea valentiae* and *Solieria robusta* 100 % seed germination was achieved in 10% concentration on the first day. The seeds treated with 10 % fresh extract of *Sargassum tenerrimum*, *Hypnea valentiae* and *Solieria robusta* showed 95 %, 85 % and 75 % germination respectively. Longer exposure to SLFs increased seed germination. Minimum % seed germination was recorded for fresh extract of *Sargassum tenerrimum* and maximum for dry extract of *Hypnea valentiae*. Similar results were also recorded for maize²⁵, green chillies and turnip²⁶, okra and tomato²⁷. When treated with 10 % extract of *Caulerpa racemosa* and *Gracilaria edulis*, *Vigna catajung* has been reported to show 100 % seed germination²⁸. Williams *et al.*²⁹ have reported gibberellin in seaweed extracts. At low concentrations gibberellin acts on seed tissue leading to increase in cell division and cell elongation, which enhance seed germination. Similar effects of gibberellin have been reported in cereals³⁰.

Root as well as hypocotyl elongation was found to be more in dry extracts than in fresh extracts made of *Sargassum tenerrimum*, *Hypnea valentiae* and *Solieria robusta* (Table 1). These observations are in agreement with earlier studies^{24, 31}. In 100 % fresh as well as dry and 70 % dry *Solieria robusta* extract and 30 %, 50 %, 70 % and 100 % *Hypnea valentiae* extract showed no hypocotyl elongation. However, in 100 % fresh *Hypnea valentiae* extract no root initiation was observed. The hypocotyl elongation could be the result of certain sugars present in the extract as suggested by Blunden and Woods³². These sugars may serve as energy source during early developmental stages of the seedling. How-

ever, presence of biological molecules like Phenylacetic acid (PAA) in the extracts might promote the hypocotyl elongation at lower concentrations³¹. Auxins, cytokinins, cis and trans ribosylzeatin, transzeatin, dihydrozeatin and N super(6) (Delta super(2)-isopentenyl)adenosine have been recorded in the seaweed extracts^{33,34}. The root as well as hypocotyl elongation has been suggested to be controlled by these hormones at lower concentrations^{35,36}. At the same time, higher concentrations of these molecules might act adversely³⁷.

Ex-situ evaluation of 10 % dry *Hypnea valentiae* extract showed three-fold increase in fresh as well as dry weight, while one- to two-fold increase in root length, shoot length and leaf area, and two-fold increase in total chlorophyll content of *Trigonella foenum graecum* plants. At lower concentrations, two- to three-fold increase in fresh weight of shoots and roots as well as leaf area was observed in *Cyamopsis tetragonoloba* when treated with dry *Caulerpa scalpelliformis* and *Gracilaria corticata* extracts³⁸. Similar observations have been made for cereals and millets³⁹.

Effortless decomposition of seaweeds provide certain kinds of essential minerals and amino acids^{40,41}. Presence of oligosaccharides in seaweed extracts and nitrate availability of organic nitrogen along with other factors such as trace metals, phyto-hormones may cumulatively augment biomass increase⁴². Enhancement in leaf pigments in plants treated with seaweed extract has also been recorded by previous workers^{23,38,39,43}.

Seaweeds, which are very abundant along Indian coast, could be best utilized for our agriculture. Increasing number of seaweed polysaccharide producing industries are opting for production of seaweed liquid fertilizers¹. Large-scale production of liquid fertilizers from this under-utilized marine renewable resource would not only help in gaining improved crop production but also provide employment to the local population.

Acknowledgement

Authors thank the Head, Department of Botany, University of Pune for providing facilities to carry out the work.

References

1. Subba Rao PV, Mantri VA. Indian seaweed resources and sustainable utilization: Scenario at the dawn of a new century. *Curr Sci* 2006; **90** : 164-174.

2. Chapman VJ. Seaweed and their uses. First Edition, London: Methuen and Co. Ltd, 1970.
3. Thivy F. Marine algal cultivation. *Salt Res Indus* 1964; **1**: 23-28.
4. Mantri VA, Chaugule BB. Less known Ethanophycological use of Seaweeds of Malvan: A survey report. *Indian Hydrobiol* 2005; **8** : 147-150
5. Mantri VA. To explore potential of some marine algae from Malvan as fertilizer, M. Sc. Dissertation, University of Pune, Pune, 1999.
6. Vijaya Parthasarathy MD, Krishnamurthy V. LSF from *Gelidiella acerosa*. In: Tewari A, Kaliaperumal N, Eswaran K, Kalimuthu S, Edwin Joseph V, editors. Seaweeds of India: Biodiversity and Biotechnology. Bhavnagar : Central Salt and Marine Chemicals Research Institute Publication, 2004. 143-145.
7. Rama Rao K. Seaweeds as biofertilizers in Indian horticulture. *Seaweed Res Utiln* 1992; **14** : 99-101.
8. Venkatraman K, Mohan VR. Effect of seaweed extract SM3 on the cyanobacterium, *Scytonema* sp. *Biomed Lett* 1994; **19** : 13-15
9. Sridhar S, Rengasamy R. Effect of seaweed liquid fertilizer obtained from *Ulva lactuca* on the biomass pigments and Protein content of *Spirulina platensis*. *Seaweed Res Utiln* 2002; **24** : 145-149.
10. Crouch IJ, Staden VJ. Evidence for the presence of plant growth regulators in commercial seaweed products. *Plant Growth Reguln* 1993; **13** : 21-29.
11. Featonby-Smith BC, van Staden J. Identification and seasonal variation of endogenous cytokinins in *Ecklonia maxima* (Osbeck) Papenf. *Bot Mar* 1984; **27** : 521-524.
12. Stephen AB, Macleod JK, Palni LS, Letham DS. Detection of cytokinin in seaweed extract. *Phytochem* 1985; **24** : 2611-2614.
13. Lingakumar K, Jeyaprakash R, Manimuthu C, Haribhaskar A. *Gracilaria edulis* - an effective alternative source as a growth regulator for legume crops. *Seaweed Res Utiln* 2002; **24** : 117-124.
14. Kingman AR, Moore J. Isolation, purification, and quantitation of several growth-regulating substances in *Ascophyllum nodosum* (Phaeophyta). *Bot Mar* 1982; **25** : 149-153.
15. Cahallen SB, Hemingway JC. Growth of higher plants in response to feeding with seaweed extracts. *Proc Int Seaweed Sympo.* 1966; **5** : 359-367.
16. van Netten C, Hoptin Cann SA, Morley DR, van Netten JP. Elemental and radioactive analysis of commercially available seaweed. *Sci Total Environ* 2000; **225** : p. 169.
17. Subba Rao PV, Mantri VA, Ganesan K. Mineral composition of edible seaweed *Porphyra vietnamensis*. *Food Chem* 2007; **102** : 215-218.
18. Ito S, Miyosshi T. Determination of Mg and Zn contents of Naruto 'wakame' *Undaria pinnatifida*. *Nippon Eiseigaku Zasshi* 1990; **45** : 795-800.
19. Darcy-Vrillon B. Nutritional aspects of the developing use of marine macroalgae for the human food industry. *Int J Food Sci Nutr* 1993; **44** : S23-S35.
20. Stephenson WM. The effect of hydrolysed seaweed on certain plant pests and diseases. *Proc Int Seaweed Sympo.* 1966; **5** : 405-415.
21. Jeannin I, Lescure JC, Morto-Gaudry JF. The effects of aqueous seaweed sprays on the growth of maize. *Bot Mar* 1991; **34** : 469-473.
22. Beckett R P, van - Staden J. The effect of seaweed concentrate on the yield of nutrient stressed wheat. *Bot Mar* 1990; **33** : 147-152.
23. Ananthraj M, Venkatesalu V. Studies on the effect of seaweed extract on *Dolichos biflorus*. *Seaweed Res Utiln* 2002; **24**: 129-137.
24. Bhosle NB, Dhargalkar VK, Untawale AG. Effect of seaweed extract on the growth of *Phaseolus vulgaris* L. *Indian J Mar Sci* 1975; **4** : 208-210.
25. Stephenson WA. Seaweed in agriculture and horticulture. Third edition, Rateaver: Peruma Valley, 1974.
26. Dhargalkar VK, Untawale AG. Some observations on the effect of seaweed liquid fertilizers on the higher plants. Proceedings of National Workshop on Algal Systems. Indian Society of Biotechnology, 1980, IIT, New Delhi.
27. Selvaraj R, Selvi M, Shakila P. Effect of seaweed liquid fertilizers on *Abelmoschus esculentus* (L.) Moench and *Lycopersicon lycopersicum* Mill. In: Tewari A, Kaliaperumal N, Eswaran K, Kalimuthu S, Edwin Joseph V, editors. Seaweeds of India: Biodiversity and Biotechnology. Bhavnagar : Central Salt and Marine Chemicals Research Institute Publication, 2004. 121-123.
28. Anantharaj M, Venkatesalu V. Effect of seaweed liquid fertilizer on *Vigna catajung*. *Seaweed Res Utiln* 2001; **23**. 33-40.
29. Williams DC, Brain KR, Blunden G, Wildgoose PB. Commercial seaweed extracts. *Proc Int Seaweed Sympo* 1981; **8** : 761-763.
30. Jacobsen JV, Chandler PM. Gibberellin and abscisic acid in germinating cereals. In: Davies PJ, editor. Plant Hormones and their Role in Plant Growth and Development, Boston: Kluwer Publication, 1987, 164-193
31. Taylor IEP, Wilkinson AJ. The occurrence of gibberellin-like substances in algae. *Phycologia* 1997; **16** : 37-42.

32. Blunden G, Woods DL The effects of aqueous seaweed extract on sugar beet. *Proc Int Seaweed Sympo* 1981; **8** : 667-672.
33. Featonby-Smith BC, Van Staden J Identification and seasonal variation of endogenous cytokinins in *Ecklonia maxima* (Osbeck) Papenf. *Bot Mar* 1984, **27** : 527-531
34. Stirk WA, Van Staden J Comparison of cytokinin- and auxin-like activity in some commercially used seaweed extracts *J Appl Phycol* 1996, **8** : 503-508
35. Esashi T, Leopold AC. Cotyledon Expansion as a bioassay for cytokinins. *Plant Physiol* 1969; **44** : 618-620
36. Huff AK, Ross CW Promotion of radish cotyledon enlargement and reducing sugar content by zeatin and red light. *Plant Physiol* 1975; **56** : 429-433
37. Beckett RP, van - Staden J The effect of seaweed concentrate on the growth and yield of potassium stressed wheat *Plant Soil* 1989, **116** : 29-36.
38. Thirumal TR, Maria Victorial Ram S, Peter Marian M Effect of seaweed liquid fertilizers on the growth and biochemical constituents of *Cyamopsis tetragonoloba* (L.) Taub. *Seaweed Res Utiln* 2003, **25** : 99-103.
39. Rajkumar IR, Subramanian SK Effect of fresh extracts and seaweed liquid fertilizers on some cereals and millets *Seaweed Res Utiln* 1999, **21** : 91-94
40. Williams SL Decomposition of the tropical macroalga *Caulerpa cupressoides* (West) C. Agardh field and laboratory studies *J Exp Mar Biol Ecol* 1984, **80** : 109-124
41. Albright LJ, Chocair J, Masuda K, Valdes M Degradation of the kelps *Macrocystis integrifolia* and *Nereocystis luetkeana* in Brithish Columbia coastal waters In Srivastana LM editor Synthetic and degradative processes in marine macrophytes, Berlin and New York Walter de Gruyter and Co, 1982. 215-231
42. Yvin J C. New approach of the mode of action of seaweed extracts in agriculture Comptes-Ren dus-der' Academie-d' Agriculture-de-France 1994, **80** : 103-112
43. Blunden G, Jenkins I, Yan - Wen L Enhanced leaf chlorophyll levels in plants treated with seaweed extract *J Appl Phycol* 1997; **8** : 535-543

Natural incidence of fusarial mycotoxins

K. NARASIMHA RAO, B. VIJAYAPAL REDDY, S. GIRISHAM and S.M. REDDY

Department of Microbiology, Kakatiya University, Warangal-506009, India.

Received November 28, 2006, Revised April 26, 2007, Accepted June 8, 2007

Abstract

An extensive and intensive survey of foods (maize and sorghum) of different regions of AP was carried out for the presence of different fusarial species and their toxins. In general all the foods analysed contained one or other species of *Fusarium*. The degree of mycotoxin contamination varied with the commodity and place of collection. Fumonisin, zearalenone, T₂ toxin, DON and DAS were some of the toxins detected.

Key words : fusarial toxins, zearalenone, T₂-toxin, fumonisins, *Fusarium* spp

Introduction

Mycotoxin contamination of foodstuffs is posing a great threat to public health due to its potent toxic nature and fairly widespread occurrence. Hardly any edible substance can be regarded as absolutely safe from mycotoxin contamination. As a matter of fact nature of toxigenic strain coupled with environmental conditions determine the incidence of mycotoxins in food commodities. During last decade several reviews have appeared on the mycotoxin contamination of food commodities from different parts of the world¹⁻³. Natural occurrence of fusarial toxins has been reported from different parts of the world⁴⁻¹⁰. However, such reports from this region are lacking. Therefore, an intensive and extensive survey of foods (sorghum and maize) both in the field and in storage, for fusarial infestation and contamination with their toxic metabolites were analysed.

Material and Methods

Maize and sorghum seed samples from three geographical regions of Andhra Pradesh were collected in sterilized polythene bags and brought to the laboratory. Condition of the samples and their place of collection

सारांश

आंध्र प्रदेश के विभिन्न क्षेत्रों में मक्का तथा ज्वार जैसे भोज्य पदार्थों पर विभिन्न फ्यूसेरियल प्रजातियों तथा इनके द्वारा उत्पन्न विषैले पदार्थों की उपस्थिति का व्यापक तथा सघन सर्वेक्षण किया गया। सामान्य रूप में सभी भोज्य पदार्थों के विश्लेषण पर, कोई न कोई फ्यूसेरियम की प्रजाति मिली है। माइकोटॉक्सिन के सदृश की डिग्री भोज्य पदार्थ तथा उसके संग्रह के स्थान के अनुरूप ही विभिन्नता दर्शाती रही है। जिन प्रमुख विषैले पदार्थों को प्राप्त किया गया उनमें से कुछ हैं, फ्यूमोनिसिन, जियरालेनोन, टी-2 विष, डान और डएस।

सांकेतिक शब्द : फ्यूसेरियल विष, जेरालीनोन, टी-2 विष, फ्यूमोनिसिन, फ्यूसेरियम स्पीसीज

were recorded carefully. Ten gm of grain sample was incubated at saturated atmosphere to induce mould growth for 7 days. At the end of incubation period the sample was mildly heated and extracted with water : acetonitrile (100 : 50) for detection of different mycotoxins. Methods of Kamimura *et al.*¹¹ and Rao *et al.*¹² were adopted for detection of different fusarial toxins.

The filtrate was extracted twice with 100 ml of chloroform. The combined extracts were then passed through anhydrous Na₂SO₄ bed to remove moisture and evaporated to dryness and redissolved in 1 ml of chloroform and spotted on TLC plates and developed in different solvent systems. Subsequently, they were dried at room temperature for 5-10 minutes and observed under long wave UV light (365 nm). The toxins were confirmed chemically by spraying the plates with different spray reagents as suggested by Kamimura *et al.*¹¹ and others¹³⁻¹⁶. Different toxins were identified with the help of colour of spot and R_f value as compared with standard data (Table 1).

Results and Discussion

Out of 141 samples of maize and sorghum collected from different regions of A.P., and analysed to

Table 1 - Detection of trichothecenes and other mycotoxins produced by *Fusarium*

Name of the toxin	Solvent system	Spray reagent	Detection	
			UV	Visible
Deoxynivalenol (DON)	C : M (97 : 3)	4,7,8	-, ch, bl	y,-,-
Diacetoxy scirpenol (DAS)	C . M (97 : 3)	6, 9	bg,-	-, br
Fusarenone - X	C : M (97 : 3)	8	bl	-
HT-2 toxin	C . M (97 : 3)	6	bg	-
Moniliformin	C : M (97 : 3)	2, 10	-	br, br
Nivalenol (NIV)	C : M (97 : 3)	4, 7, 8	-, chl, bl	y,-, -
T-2 Toxin	C : M (97 : 3)	6,9	bg,-	-,P
Zearalenone	C . M (97 : 3)	1, 2, 3, 5, 7, 8	-, -, -, br, ch, bl	br, do, ip, -, -, -
Fumonisin (FBI, FB2)	W : M (3 : 1)	4,11		br

Solvents : C = Chloroform; M = Methanol, W - Water

Spray reagents : 1 = $\text{Ce}(\text{SO}_4)_2$ 1% in 6N. H_2SO_4 ; 2 = 2,4-DNP; 3 = FeCl_3 3% in ethanol; 4 - p-anisaldehyde;
 5 - 50% H_2SO_4 in methanol; 6 = 20% H_2SO_4 ; 7 = H_2SO_4 ; 8 = 20% AlCl_3 ;
 9 = Chromatropic acid; 10 = 0.1% methanolic ninhydrin; 11 = Ninhydrin

Detection colours : bl = Blue; ch = Charring, y = Yellow; bg = Blue green, br = Brown; p = Purple;
 do = Dark orange; lp = light purple; pi = Pink; bb = Bright blue.

get 52 samples contaminated with one or other fusarial toxins. Some of the samples were contaminated with more than one mycotoxin. Out of 8 samples of sorghum collected from Khammam, 4 were infested by either *F. moniliforme* Sheldon, *F. oxysporum* Schlecht or *F. equiseti* Corda. One sample was contaminated by fumonisins B₁, ZEA and DAS, while others were contaminated with ZEA, T-2 and DON. The samples infested with *F. equiseti* Corda. were found to contain ZEA and DON. Out of 6 samples of maize collected from Khammam were infested by 3 species of *Fusarium* and 2 samples were found to be contaminated with their respective toxins. One sample was contaminated with fumonisins, ZEA and T-2 toxin, while other samples were infested by both *F. oxysporum* Schlecht and *F. solani* Mourt. In this sample ZEA, T-2, DON and DAS could be detected. Out of 12 and 8 samples, 5 and 3 of sorghum and maize, respectively were infested by *F. moniliforme* Sheldon, *F. oxysporum* Schlecht, *F. acuminatum* Ellis and Everth and *F. equiseti* Corda. One sample was contaminated by fumonisins, ZEA, DON and DAS, while other samples were contaminated with DON only. Rest of the samples were contaminated with

ZEA, T-2, DON and DAS. Out of 7 samples of sorghum collected from Karimnagar 3 samples were infested by *F. moniliforme* Sheldon and *F. oxysporum* Schlecht. One sample was contaminated by ZEA and DON, the other two samples were contaminated by fumonisins B₁, B₂ and T-2 toxin. Out of 5 samples of maize, one sample was infested by *F. equiseti* Corda but none of the mycotoxins could be detected. One sample out of 5 samples of sorghum collected from Nalgonda was infested by *F. moniliforme* Sheldon and *F. heterosporum* Nees and contained fumonisins B₁ and ZEA. Two samples of maize, out of 5 collected from Adilabad, were contaminated by fumonisins B₁, zearalenone T-2 toxin DON, DAS and supported the growth of *F. moniliforme* Sheldon. Out of 6 samples of sorghum collected from Adilabad, only two samples were contaminated either by zearalenone or DON, while out of 8 samples of maize, 3 were contaminated by fumonisins, zearalenone, T-2 toxin and DAS. Two samples of sorghum out of 7 samples, collected from Nizamabad were contaminated by fumonisins, zearalenone and T-2 toxin. Similarly 2 out of 6 samples of maize were contaminated by zearalenone, T-2 toxin and DON. None of the 3

Table 2 - Natural incidence of fusarial mycotoxins in sorghum and maize kernels.

Place of collection	Name of the commodity	No. of samples screened	No. of positive samples	Name of the fungus	Name of the toxin					
					FB1	FB2	ZEA	T-2	DON	DAS
[1]	[2]	[3]	[4]	[5]	[6]	[7]	[8]	[9]	[10]	[11]
Adilabad	Sorghum	6	2	<i>F. moniliforme</i>	--	--	+	--	+	--
				<i>F. oxysporum</i>	--	--	+	--	+	--
				<i>F. equiseti</i>	--	--	+	+	--	--
	Maize	8	3	<i>F. moniliforme</i>	+	--	+	--	--	+
				<i>F. oxysporum</i>	--	--	+	+	--	--
				<i>F. semitectum</i>	--	--	--	+	--	+
Cuddapah	Sorghum	2	1	<i>F. moniliforme</i>	--	--	--	--	--	--
	Maize	4	3	<i>F. moniliforme</i>	+	--	--	--	--	--
				<i>F. solani</i>	--	--	+	--	--	--
				<i>F. semitectum</i>	--	--	+	+	--	--
E. Godavari	Sorghum	8	3	<i>F. moniliforme</i>	+	+	--	--	--	--
				<i>F. solani</i>	--	--	+	+	--	+
	Maize	12	5	<i>F. moniliforme</i>	--	--	+	--	--	--
				<i>F. acuminatum</i>	--	--	--	--	--	+
Karimnagar	Sorghum	7	3	<i>F. moniliforme</i>	+	+	--	+	--	--
				<i>F. oxysporum</i>	--	--	+	--	+	--
	Maize	5	1	<i>F. equiseti</i>	--	--	--	--	--	--
Khammam	Sorghum	8	4	<i>F. moniliforme</i>	+	--	+	--	--	+
				<i>F. oxysporum</i>	--	--	+	+	+	--
				<i>F. equiseti</i>	--	--	+	--	+	+
	Maize	6	2	<i>F. moniliforme</i>	+	--	+	+	--	--
				<i>F. solani</i>	--	--	+	--	+	--
				<i>F. oxysporum</i>	--	--	+	+	--	+
Nalgonda	Sorghum	5	1	<i>F. semitectum</i>	--	--	+	+	+	--
				<i>F. moniliforme</i>	+	--	+	--	--	--
	Maize	5	2	<i>F. heterosporum</i>	--	--	+	+	--	+
				<i>F. moniliforme</i>	+	--	+	+	+	+
Nizamabad	Sorghum	7	2	<i>F. heterosporum</i>	--	--	+	--	--	--
				<i>F. moniliforme</i>	+	--	+	--	--	--
				<i>F. oxysporum</i>	--	--	--	+	--	--

[1]	[2]	[3]	[4]	[5]	[6]	[7]	[8]	[9]	[10]	[11]
				<i>F. equiseti</i>	--	--	+	+	--	--
	Maize	6	2	<i>F. moniliforme</i>	+	--	--	+	--	--
				<i>F. oxysporum</i>	--	--	+	--	+	--
				<i>F. semitectum</i>	--	--	+	+	--	--
Prakasham	Sorghum	3	2	<i>F. moniliforme</i>	+	--	+	--	--	--
				<i>F. oxysporum</i>	--	--	--	+	--	+
				<i>F. acuminatum</i>	--	--	+	+	--	--
	Maize	5	2	<i>F. moniliforme</i>	+	--	--	+	+	--
				<i>F. oxysporum</i>	--	--	--	--	+	--
				<i>F. semitectum</i>	--	--	+	--	--	--
Rangareddy	Sorghum	3	1	<i>F. moniliforme</i>	--	--	--	--	--	--
	Maize	4	1	<i>F. moniliforme</i>	+	+	+	--	--	--
				<i>F. oxysporum</i>	--	--	--	+	--	--
				<i>F. solani</i>	--	--	+	--	--	+
Warangal	Sorghum	12	5	<i>F. moniliforme</i>	+	--	+	--	+	+
				<i>F. oxysporum</i>	--	--	+	+	--	+
				<i>F. acuminatum</i>	--	--	--	--	+	--
	Maize	8	3	<i>F. moniliforme</i>	+	+	+	--	--	+
				<i>F. oxysporum</i>	--	--	+	--	+	+
				<i>F. equiseti</i>	--	--	+	--	+	--
W. Godavari	Sorghum	5	2	<i>F. moniliforme</i>	--	+	--	--	--	--
				<i>F. semitectum</i>	--	--	--	--	--	--
	Maize	9	4	<i>F. moniliforme</i>	+	--	--	--	--	--
				<i>F. oxysporum</i>	--	--	+	+	--	--
				<i>F. solani</i>	--	--	+	--	--	--

samples of sorghum collected from Ranga Reddy were found to be show fusarial toxin contamination but they were infested with *F. moniliforme* Sheldon. Out of 4 samples of maize one sample was found to contain fumonisins, zearalenone, T-2 toxin and DAS and supported the growth of *F. moniliforme* Sheldon. Similarly one out of 2 samples of sorghum collected from Cuddapah was found to be infested by *F. moniliforme* Sheldon but contained no mycotoxin, while out of 4 samples of maize 3 were infested by *F. moniliforme* Sheldon, *F. solani* Mourt and *F. semitectum* Berk and Ravenel and contained zearalenone and T-

2 toxin. *F. moniliforme* Sheldon was found to produce fumonisins B₁ and B₂ while other samples contained zearalenone and T-2 toxin. Two out of 3 samples of sorghum and 4 and 5 maize samples collected from Prakasham were infested by *F. moniliforme* Sheldon, *F. oxysporum* Schlecht and *F. acuminatum* Ellis and Everth and produced fumonisins, zearalenone, DON and DAS. Out of 8 samples of sorghum collected from East-godavari, 3 samples were infested by either *F. moniliforme* Sheldon or *F. solani* Mourt and contained fumonisins, T-2 and DAS. Five out of 12 samples of maize were contaminated by either zearalenone or DAS

and infested by either *F. moniliforme* Sheldon or *F. acuminatum* Ellis and Everth. Out of 5 samples of sorghum collected from West-godavari, 2 were contaminated by fumonisins B₂ only and infested by *F. moniliforme* Sheldon and *F. semitectum* Berk and Ravenel. Out of 9 samples of maize 4 were contaminated by one or other mycotoxins and infested by *F. moniliforme* Sheldon, *F. oxysporum* Schlecht or *F. solani* Mourt.

Acknowledgements

Thanks are due to Head, Department of Microbiology and UGC for providing necessary facilities and financial assistance respectively.

References

1. Steyn PS. Mycotoxins general view, chemistry and structure. *Toxicol Lett* 1995; **82** : 843-851.
2. Yoshizawa T, Gao HP. Further study on *Fusarium* mycotoxins in corn and wheat from a high-risk area for human esophageal cancer in China. *Mytoxins* 1997; **45**: 51-55.
3. Perkowski T, Jelen H, Kiecana I, Golinski P. Natural contamination of spring barley with group A trichothecene mycotoxins in South-eastern Poland. *J Food Contam* 1997; **14** : 321-325.
4. Pacin A, Resnik SL, Nejra MS, Molto G, Martinez EJ. Natural occurrence of Deoxynivalenol in Wheat flour and bakery products in Argentina. *J Food Add and Contam* 1997; **14** : 327-331.
5. Pellet A. Natural occurrence of mycotoxins in foods and feeds - An update review. *Revue Medicine Veterinaire* 1998; **149** : 479-492.
6. Jeong-Ahseo, Yin-Wom Lee. Natural occurrence of the 'C' series of fumonisins in mouldy corn. *Appl Environ Microbiol* 1998; **64** : 1331-1334.
7. Scudamore A, Susan Patel. Survey of aflatoxins, ochratoxin A, zearalenone and fumonisins in maize imported into the United Kingdom. *J Food Add Contam* 2000; **17** : 407-416.
8. Sugiura Y. Cereals and zearalenone producing *Fusarium* species. *Mycotoxins* 2000; **50** : 125-129.
9. Josephs RD, Schumacher R, Krishna R. International inter laboratory study for the determination of the *Fusarium* mycotoxins zearalenone and deoxynivalenol in agricultural commodities. *J Food Add and Contam* 2001; **18** : 417-430.
10. Silva Vargas. A survey of zearalenone in corn using Ramer myeasap 224 column and high performance liquid chromatography. *J Food Add and Contam* 2001; **18** : 39-45.
11. Kamimura H, Nishijima M, Yasuda K, Saito K, Ibe A, Nagayama J, Oshiyama H, Naoi Y. Simultaneous detection of fusarial toxins. *J AOAC* 1981; **64** : 1067-1073.
12. Rao GV, Rao PS, Girisham S, Reddy SM. A novel spray reagent for chromatographic detection of trichothecene toxins. *Curr Sci* 1985; **54** : 507-509.
13. Ramakrishna Y, Bhat RV. Comparison of different spray reagents for identification of trichothecenes. *Curr Sci* 1987; **56** : 524-526.
14. Martin Steyn, Thiel PG, Vanschalkwyk GC. *J Assoc Off Anal Chem* 1978; **61** : p. 576.
15. Mirocha CJ, Schauerhames B, Pathre SV. Isolation, detection and quantitation of zearalenone in maize and barley. *J Assoc Off Anal Chem* 1974; **57** : 1104-1110.
16. Sydenham EW. The chromatographic determination of *Fusarium* toxins in maize associated with human/oesophageal cancer. M.Sc. thesis. University of Capte Town, South Africa, 1989.

Promotion and cultivation of *Thysanolaena maxima* (Roxb.) Kuntze : A multiuse species in Uttarakhand

ANWESHA SAH* and UMA T. PALNI

Department of Botany D.S.B. Campus, Kumaun University, Nainital-2631002 India.

*e-mail : anweshar200017@yahoo.com

Received December 5, 2006, Revised June 29, 2007, Accepted August 6, 2007

Abstract

The natural resources form major components of life-support system in hilly areas. They include forests, livestock, non-timber forest produce, agricultural and grazing lands etc. Livestock plays a major role in sustaining the life-support system in the region that largely operates at subsistence levels. The rural community - particularly women in Uttarakhand, bear heavy burden as they are entrusted with various indoor and outdoor tasks, such as collection of water, fuelwood and fodder from long distances. The availability of fodder has qualitative, quantitative and technological constraints. Domestication of *Thysanolaena maxima* (Roxb.) Kuntze (broom grass), a multiuse species of high fodder value, by the rural communities is suggested as a solution. Nurseries of this grass were established at Nainital and different villages within the district to promote it as a nutritious fodder and to show its potential value as 'biological screen', 'potted house plant', and for production of 'brooms' for household use, and also as a plant of medicinal use. The adoption of this economically important grass can reduce the drudgery of rural women of Uttarakhand by strengthening their financial condition. For its popularisation amongst villagers, a number of workshops, and awareness programmes were conducted and publicity material was also produced and distributed to the rural folk of this region.

Key words: broom grass, central Himalaya, fodder, cottage industry

Introduction

The major components of life-support system in the hilly areas are livestock, forests, agricultural and grazing lands. These components form an intricate web and livestock play a major role in sustaining the inter-linkages. Almost every family maintains some livestock, consisting of cows, oxen, goats and buffalo also. Besides contributing to a balanced diet and some income through the sale of extra produce, livestock supports crop production by providing much needed farm power and manure, vital for the prevailing organic agriculture in

सारांश

पहाड़ी क्षेत्रों में जीवन निर्वाह विधियों के मुख्य तत्वों में प्राकृतिक ससाधनों के रूप में जंगल, मवेशी, गैर इमारती लकड़ी वाले वन का उत्पादन, कृषि और चारागाह भूमि सम्मिलित हैं। इन स्थानों पर जीवन-यापन विधियों में मवेशी मुख्य भूमिका निभाते हैं। ग्रामीण वर्ग में, मुख्यतः उत्तराखण्ड क्षेत्र की महिलाओं को घर के अंदर एवं बाहर के कार्यों, जैसे पानी एकत्र करना, ईंधन हेतु दूर-दूर से लकड़ी एवं चारा लाने के कारण अत्यधिक बोझ सहन करना पड़ता है। चारे की प्राप्ति में गुणवत्ता, मात्रा तथा तकनीकी बाधाएं आती हैं। *थायोसैनोलीना मैक्सिमा* (राक्ज) कुट्ज (झाड़ू घास) की बहुउपयोगिता एवं उच्च पोष्टिक चारे के कारण ग्रामीण वर्ग द्वारा इसका घरेलू उत्पादन एक विकल्प के रूप में महत्वपूर्ण है। इस घास को पोष्टिक चारे के रूप में, जैविक स्क्रीन तथा गमले में घरेलू पौधे के रूप में, झाड़ू बनाने तथा दवा के पौधे के रूप में, बढ़ावा देने हेतु, नैनीताल एवं आसपास के गांवों में नर्सरी के रूप में स्थापित किया गया है। इस बहुउपयोगी घास का घरेलू उत्पादन उत्तराखण्ड की महिलाओं के कार्य बोझ एवं आर्थिक दशा को सुदृढ़ करेगा। इसे परिचरित करने हेतु अनेक कार्यशालाएं, जागरूकता कार्यक्रम ग्रामीणों हेतु चलाये गये हैं तथा प्रचार सामग्री का वितरण इस क्षेत्र के ग्रामीण वर्ग में किया गया है।

सांकेतिक शब्द : झाड़ू घास, मध्य हिमालय, चारा, घरेलू उद्योग

the hills. The support of agronomic production per ha of cropland requires the net production (in terms of green leaves for animal fodder, dry leaves for mulch, and wood for cooking) from 15 ha of well-stocked forest^{1,2}. However, presently only 2 ha of forest is supporting 1 ha of agriculture in Uttarakhand.

Women and young girls of rural areas in the hills, who are responsible for fodder collection, in addition to water and fuelwood collection, have to carry these "necessities" as headloads for long distances and are the worst affected. The women are heavily engaged

in agricultural and non-agricultural activities for 7-8 hours per day throughout the year³. Various agricultural operations, viz., field preparation, yield protection, yield enhancement and yield collection, account for about 60% of the total expended energy of women in the age group of 16-35 years and about 23% in animal care activities, such as cattle care during free-range grazing, stall feeding and in fodder and leaf litter collection⁴.

The present work was carried out to explore the possibility of increasing the use of a multi-utility species, *Thysanolaena maxima* (Roxb.) Kuntze (broom grass), for reducing the drudgery of rural women in the hills by enhancing on-farm availability of fodder, and economic gains from the sale of brooms and other products prepared from this plant. The fodder obtained from *T. maxima* leaves is of good quality with digestibility at about 58%, crude protein in the range of 9.45%-18.07% and crude fibre at about 30%^{5, 6, 7}. Further, the potential role of *T. maxima* in conserving soil and water under different land-use systems has also been recognized^{8, 9}.

Material and Methods

The study was carried out in the vicinity of Nainital town in Kumaun Himalaya with sites located between 343-2136 m amsl. Stock material (rhizomes) of *T. maxima* was obtained from plants growing in wild at Ghatgarh and Bhimtal areas in Uttarakhand.

Development of nurseries and transplantation: A mixture of farm yard manure (FYM), soil and sand (in the ratio of 1:1:1) was used for preparing nursery beds in the experimental sites at Bhimtal (1575 m amsl), Khurpatal (1627 m amsl) and Chilkiya (343 m amsl). The rhizomes were treated with a solution of Bavistin (1.5%, w/v) for four hours before plantation. The propagules were planted at a plant-to-plant distance of 24 cm. One year old plants raised in the nurseries were transplanted, with one plant m⁻² density, at three different sites near the homes of voluntary farmers (Bhimtal, Khurpatal and Chilkiya) for field trials in the monsoon season of 2004, and also just before the onset of winter, later in the year.

Biomass removal experiments: These experiments included two different treatments. For these experiments, ten clumps were taken for each category of clipping.

- i. In the first experiment, 15-months old plants were clipped twice at three different heights, first in October, 2004 and again in April, 2005.
 - a. 20% of the total plant height
 - b. 40% of the total plant height
 - c. 60% of the total plant height
- ii. In the second experiment, a certain proportion of culms were removed from the base of the plant, in October, 2004 and April, 2005,
 - a. 25% of the total number of culms in the plant
 - b. 50% of the total number of culms in the plant

The new growth that followed after treatments was harvested after six months and the data were recorded in terms of culm number, culm height, leaf/culm, belowground and total biomass, etc.

Drudgery reduction: The mean fresh weight of *T. maxima* leaf, mean number of leaves per plant and the number of plants per hectare were considered along with two levels of the harvest.

Income generation: For determining the income generated from the sale of brooms made out of *T. maxima* panicles, the cost of raw materials involved, the labour inputs and the market price of brooms were considered.

Awareness programmes: Information leaflets on the multiple uses and propagation of broom grass were developed and printed in Hindi and English for distribution among the common masses during orientation/training workshops.

Results

Growth performance of *T. maxima* at three nurseries

The data recorded from 24 months old *T. maxima* plants indicated best growth performance at Khurpatal, followed by Chilkiya in terms of culm and leaf numbers, and culm height, while in terms of leaf area it was best at Bhimtal (Fig. 1). The soil analyses revealed higher organic matter and nitrogen contents at Khurpatal as compared to other sites. Judging by the highest leaf area attained by the plants, the nursery at Bhimtal could have resulted in best overall performance, had the plants not been damaged by the severe winter frost. The learning

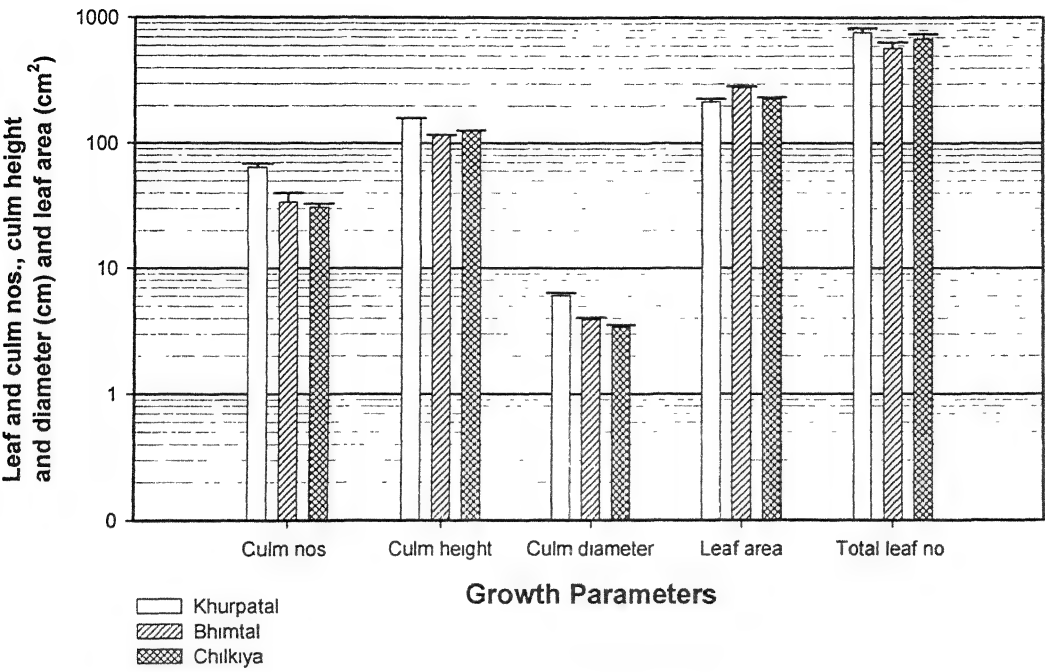


Fig. 1- Comparison of growth parameters of *T. maxima* at three unprotected (open) experimental sites. The values represent mean of 10 plants. The values on y-axis are represented on \log_{10} scale

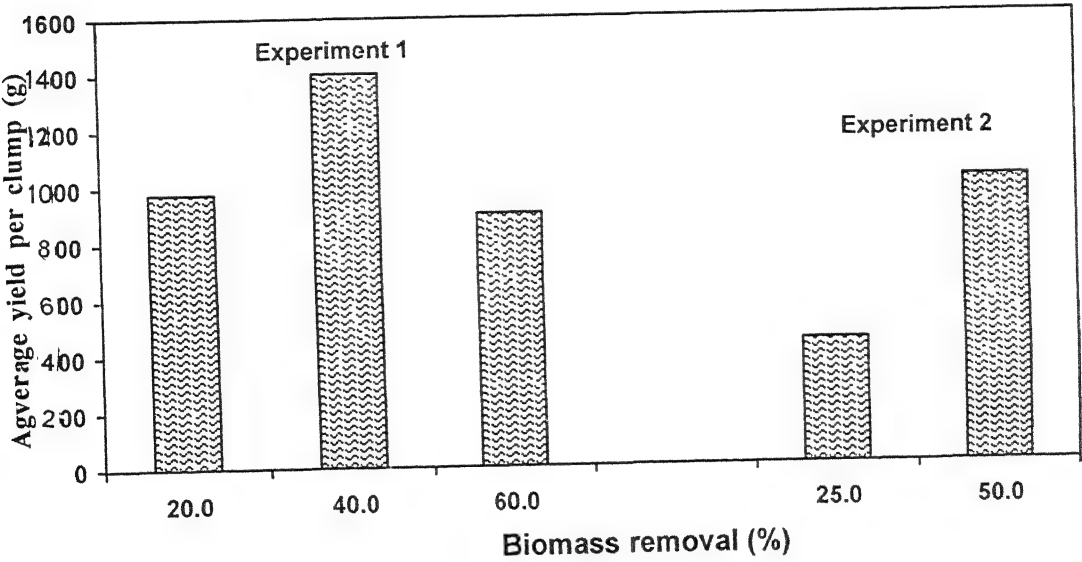


Fig. 2 - Comparison of yield per clump (g) from different clipping experiments. Data were recorded after 6 months.

lesson from this is to harvest above ground biomass well before the onset of winter frost.

Biomass removal experiments

ANOVA showed that there was significant ($P < 0.05$) difference in the amount of leaves per plant (no. of leaves per culm \times no. of culms) across different treatments and across the years of study ($P < 0.01$). The first experiment showed that 40% clipping yielded best results, though yields were also reasonable with 60% clipping (Fig. 2). In the second experiment, 50% removal of culms yielded best results. Since among the treatments, 40% clipping gave the best results, this could be recommended as a practice when the plant is used as a source for green fodder.

From these experiments, the yield obtained at 40% and 60% level of harvest indicates that if broom grass is grown in one hectare of land, close to a village, it can save rural women up to 290 headloads (30 kg per headload as observed in the present study) of fodder collection per person in a year. This would provide sufficient fresh fodder (@20 kg per animal per day (as observed in the present study) for one milch animal for about 10 months in a year. The basic data for above calculations are summarised in Table 1.

Table 1 - Yield of fodder leaves from *T. maxima*

Parameter	Khurpatal	Chilkiya
Average number of leaves per plant	761.00	675.33
Average fresh weight of a leaf in g.	1.90	1.90
Average no. of fresh leaves per plant	1445.90	1283.13
Average weight of fresh leaves in kg	1.45	1.28
Fresh weight of leaves @ 40% harvest	0.578	0.513
Fresh weight of leaves @ 60% harvest	0.868	0.770
Average no. of plants per ha	10000	10000
Fresh weight of leaves in tonnes per ha @ 40% harvest	5.78	5.13
Fresh weight of leaves in tonnes per ha @ 60% harvest	8.68	7.70

Income Generation

The entrepreneur earning from the sale of brooms purchased *T. maxima* panicles from various people (who collected them from forests) at the rate of Rs. 350.00 per bundle that provides raw material for 90-100 brooms.

The entrepreneur also purchased plastic sleeves for making the broom handles and a polythene bags for packing the prepared brooms from Moradabad (a town in U.P. about 100 km from the village). The total cost of production was about Rs. 7-8 per broom and it was sold in the Ramnagar market at a price of Rs. 15-18 depending upon its weight. This activity yielded the entrepreneur an additional income of Rs. 7000-10000 seasonally.

Awareness programmes

A number of visits were made to various sites for the collection of data and interaction with the villagers. 'Mahila Mangal Dal', 'Ekta Swayam Sahayata Sangh' and 'Sainiaon ka Sangh' were the major women self-help groups that were enthusiastic to adopt the cultivation of *T. maxima* in their villages. Workshops and meetings were held in villages located in the hilly and bhabar regions of Kumaun, Uttarakhand. In these workshops, the target audience were mainly women because introduction of *T. maxima* would be of most benefit to them in terms of reduction of drudgery in fodder collection and for income generation through the sale of brooms. During the workshop, techniques of broom grass propagation were demonstrated and some rhizomes/slips (planting material) of this multiuse plant were distributed to interested participants. In addition to this, leaflets prepared for the project were distributed to women and other participants for reference.

Discussion

Thysanolaena maxima (Roxb.) Kuntze is being used in bioengineering works in some parts of Central Himalaya through planting of rhizome raised plants in diagonal rows^{8, 9}. Its main function in bioengineering is to shield, catch and to provide reinforcement to the sliding soil^{5, 10}. The grass is also grown as an ornamental potted plant as well as an effective biological screen in the gardens. This plant is also known for its medicinal uses. Decoction of roots is used as a mouthwash during fever. A paste of dried or fresh roots is applied on the skin to check boils¹¹. The traditional healers of Bastar region use the decoction of whole herb internally for many purposes. It is used by healers as a remedy in the treatment of high fever¹². The traditional healers of Pendra region suggest the patients having the problem of bavasir (piles) to wash the anus with this decoction¹³.

During the present project, work of an entrepreneur in Village, Sawaldeo of Block Ramnagar, District Nainital (Uttarakhand) who runs an enterprise based on the production and sale of brooms was also studied. It was found that his venture yields earnings of about Rs. 7000-10000 per year. The brooms are in much demand in the urban and semi-urban areas of Uttarakhand and are at present bought from Uttar Pradesh to be sold by the retail shopkeepers in the region. Thus, there is considerable potential of *T. maxima* as an income generating activity, based on the sale/making of brooms/potted plants, etc. for rural women folk of Uttarakhand. The results of the clipping experiments clearly indicate that this grass yields good quality green fodder for milch animals, especially during the lean period.

Thus, this multiuse species, *T. maxima*, has the potential of providing supplemental income to the womenfolk of Uttarakhand besides reducing their workload in terms of fodder collection. The following income generating activities need to be promoted: (i) sale of brooms made from this grass, (ii) sale of surplus milk due to increased production by feeding good quality of fodder to the milch animals, (iii) sale of (a) potted plants for decorative purposes, and (b) slips for creating biological screens, (iv) sale of propagules for use by other farmers, and (v) sale of various components for use in traditional medicines.

Acknowledgements

Science and Society Division of D.S.T., Government of India is thanked for financial support for this project. Guidance received from Drs. L.M.S. Palni and Sanjeev Bhuchar is acknowledged.

References

1. Pandey Uma, Singh JS. Energy-flow relationships between agro- and forest ecosystems in Central Himalaya. *Environmental Conservation* 1984; 11(1) : 45-53.
2. Singh JS, Pandey Uma, Tiwari AK. Man and Forest: A Central Himalaya Case Study. *Ambio* 1984; 12(2) : 80-87.
3. Khanka SS. Labour Force, employment and unemployment in a backward economy. New Delhi: Himalaya Publishing House, 1998
4. Bisht BS, Bhuchar S, Bisht SS, Kothiyari BP, Palni LMS. *J Human Ecol* 2000, 11(6) : 487-489.
5. Palni LMS, Kothiyari BP, Rikhari HC, Bhuchar S, Negi GCS, Sharma E, Samant SS, Bisht S, Choudhury D. *Thysanolaena maxima* (Roxb.) Kuntze: A multipurpose perennial grass of high fodder value. *Himalaya Paryavaran* 1994, 6(1) : p. 9.
6. Singh KA, Rai RN, Balaramman N, Singh LN. Evaluation of forage grasses for mid hills of Sikkim (Eastern Himalaya). *Range Management and Agroforestry* 1995, 16 : 39-45
7. Huque KS, Rahman MM, Jalil MA. Nutritive Value of Major Feed Ingredients, Usually Browsed and Their Responses to Gayals (*Bos frontalis*) in the Hill Tract Area. *Pakistan J Biol Sci* 2001; 4(12) : 1559-1561
8. Agrawal DK, Rikhari HC. Mountain risk engineering, low cost physical and biological measures for control of small hill slope instability. In: Research for Mountain Development: Some initiatives and accomplishments. Nainital Gyanodaya Prakashan, 1998; 114-119
9. Bhuchar SK. An Eco-Physiological Evaluation of *Thysanolaena maxima* (broom grass). A Multipurpose, Perennial Grass of High Fodder Value. Ph D Thesis, Kumaun University, Nainital, 2000.
10. Uma Shanker R, Lama SD, Bawa KS. Ecology and economics of domestication of non-timber forest products: An illustration of broomgrass in Darjeeling Himalaya. *J Trop For Sci* 2001, 13 : 171-191
11. Rai LK, Sharma E. Medicinal Plants of the Sikkim Himalaya. Status, Usage and Potential. New Delhi: Vedams e-Books (P) Ltd, 1994
12. Oudhia P. Traditional medicinal knowledge about herbs used in treatment of cancer in Chhattisgarh, India. VII. Interactions with female traditional healers. *Non Wood News* 2007, 14 : p. 79.
13. Oudhia P, Pal AR, Pali GP. Traditional medicinal knowledge about common crop weeds in Bagbahera (India) region: A survey. *Agric Sci Digest* 2002; 22(1) : 53-54.

Environmentally affected allelopathic response of a few weeds on late blight disease of potato

S.N. PHUKAN

Department of Botany, North Lakhimpur College, Assam-787031, India.

Received March 7, 2006, Revised January 29, 2007, Accepted January 31, 2007

Abstract

The course of development of late Blight disease of potato was studied in experimental plots planted with seed tubers of cultivar Khasigaro, treated with extracts of weed flora namely, *Ludwigia purviflora* Roxb. *Polygonum plabejum* R. Br. and *Anisomeles ovata* Br. During two crop seasons 2004-05 and 2005-06, maximum infection of plants occurred during rainy days with moderately low temperature and high humidity prevailing within the plant canopy in both treated and untreated plants. The incidence of late Blight was found to be significantly delayed and percent Blight intensity sufficiently decreased when the plants were treated with extracts of *Ludwigia* and *Anisomeles*. Complete eradication of the disease in the plants treated with the weed extracts was, however, not possible. Inhibition of growth of *Phytophthora infestans* was also evident with the weed extracts during *in-vitro* studies.

Key words : potato crop, allelopathic response, weeds, environmental factors.

Introduction

The potato crop is affected by several fungal diseases causing heavy loss in the yield. The late blight alone causes 70-80 percent loss in potato crop¹. The influence of environmental factors on the development of late blight disease has been emphasized by many workers. Couzens and Evans² stated that under appropriate climatic conditions, infected tubers give rise to initial foci of infection, the dimension, frequency and persistence of which vary from season to season and from place to place. Control of late blight disease has been a major issue before the workers, as till date full eradication of the pathogen by the fungicides, has not been confirmed. Now a days, in view of the toxicity caused by the chemicals during cultivation and storage, eco-friendly strategies, such as use of plant products, parasitoids, predators and pathogens³ are being adopted. Certain metabolites of several higher plants are known

सारांश

आलू में 'लेट ब्लाइट रोग' के विकास की गति का अध्ययन 2004-05 तथा 2005-06 के उपज काल में कुछ प्रयोगात्मक भूखण्डों में 'खासीगारो' नस्ल के बीजकंदों को उगाने के बाद, *लड्वीजिया पर्वीफ्लोरा* राक्सब, *पालीगोनियम प्लेबेजम* आर बीआर तथा *एनीसोमेलिस ओवेटा* ब्रा जैसे खरपतवारों के सार तत्व द्वारा प्रतिपादित कर के, किया गया। उपचारित तथा अनुपचारित दोनों प्रकार के पौधों में अत्यधिक सक्रमकता बरसात के दिनों में दिखी, जब तापमान समुचित रूप में कम तथा आद्रता अधिक थी। ऐसी परिस्थिति में जब पौधों को *लाइवीजिया* तथा *एनीसोमेलिस* के सारतत्व द्वारा प्रतिपादित कर दिया गया, 'लेट ब्लाइट रोग' का आपतन सार्थक रूप में विलम्बित दिखा, साथ ही साथ ब्लाइट की तीव्रता का प्रतिशत भी पर्याप्त रूप में कम था। खरपतवार के सार तत्व द्वारा प्रतिपादित पौधों में इस रोग का पूर्ण निस्तारण संभावित नहीं था। ऐसे पौधों के अंतर्गत अध्ययन द्वारा *फाइटोफ्थोरा इनफेस्टेंस* वृद्धि का निषेध सुस्पष्ट था।

सांकेतिक शब्द : आलू की उपज, एलीलोपैथिक प्रतिक्रिया, खरपतवार, पर्यावरणीय कारक

to produce toxic effects against spore germination and mycelial growth of phytopathogenic fungi⁴. The present study aims at investigating the pattern of incidence and development of late blight disease of potato crop as affected by climatic conditions of Assam using tubers treated with some common weed plant extracts available in the potato cultivated areas.

Material and Methods

1. The study was made in four thoroughly ploughed experimental plots, measuring 5m long and 4m wide, making three replications. Healthy potato seed tubers were hand planted in the base of ridges of rows 70cm apart with 20cm spacing in between the tubers. A total of 120 seed tubers of average size of potato cultivar 'Khasigaro' were planted in each plot. After the plants attained 7cm height, earthing was done. The plants were examined from time to

time for the appearance of late blight by visual estimation⁵. A few seed tubers which did not germinate were discarded.

2. Relative humidity, temperature and rainfall data were recorded during the entire period of investigation.

3. Root, leaf and stem extracts from three fern plants namely, *Ludwigia purviflora* Roxb., *Polygonum plebejum* R.Br. and *Anisomeles ovata* Br. were prepared by the method followed by Parihar and Bohra⁶. The above plant species were selected because they grow profusely in the potato cultivation areas of Assam and also utilized by the native tribes for medicinal purposes. 5 g of fresh plant parts were washed 2-3 times with tap water and then surface sterilized with 90% alcohol. Subsequently, the material was grounded in 50ml acetone. The acetone macerates were kept for 24h at room temperature, to let the acetone evaporate. In the remaining residue, 50ml of distilled water was added. Macerates were squeezed through double layered muslin cloth and filtered through filter paper. After filtration, aliquots were centrifuged at 10,000 rpm for 20 min. The supernatant was filtered through Whatman No. 1 filter paper and then sterilized by passing through 0.2 micron disposable filters. The extracts (10%) thus obtained were used for the studies. Aquous plant parts extracts were simultaneously prepared by using distilled water.

4. The antifungal activity of the plant extract on the mycelial growth of *Phytophthora infestans* was

measured by adding 1ml extract to 10ml 'Limabean Agar media' (Savage *et al.*⁷). A small portion of sporulating colony was placed in the center of the petridishes containing the culture media. Diameter of mycelial mats were measured after 72 hours. The rate of spore germination was evaluated by employing spore germination tests in cavity slides (Chandrol and Karkum⁸). Spores were collected by gently brushing the surface of infected leaf, with a sterile needle, suspended in distilled water. For each treatment, one drop of extract with loopful of spore suspension was put in cavity slides and covered with cover slip. Suspensions prepared in a similar manner in sterile water served as control. After incubation for 12 hours at 18°C, at least six microscopic fields were examined to obtain germination percentage by using the following formula.

$$\text{Percentage of spore germination} = \frac{\text{Total number of germinated spores} \times 100}{\text{No of spores/loop in spore suspension}}$$

Results and Discussion

The antifungal property of the extracts of the test plants, studied as a part of the present experiment revealed significant influence of the extracts on the mycelial growth and sporulation of the causal organism of late blight disease *Phytophthora infestans*. A significant reduction in mycelial growth was noticed when treated with acetone extract of *Ludwigia purviflora* (Roxb.)

Table 1 - Effect of plant extract on mycelial growth (mm) and germination of sporangia (%) of *P. infestans*.

Plant extract		Control		Root		Stem		Leaf	
		A	B	A	B	A	B	A	B
		18.4	78.2	-	-	-	-	-	-
T ₁	Aquous	-	-	08.3	20.6	12.3	33.4	10.2	24.4
	Acetone	-	-	05.3	14.5	09.6	32.4	04.3	21.6
T ₂	Aquous	-	-	12.6	34.6	16.5	57.8	15.4	47.8
	Acetone	-	-	13.0	47.2	12.4	49.6	15.9	53.2
T ₃	Aquous	-	-	09.2	32.3	05.4	57.8	09.3	27.4
	Acetone	-	-	06.3	39.3	17.9	73.2	07.5	19.3
C.D. at 5%		T ₁ =0.03		T ₁ = <i>Ludwigia purviflora</i>		A= Mycelial growth			
		T ₂ = 0.06		T ₂ = <i>Polygonum plebejum</i>		B = Germination of sporangia			
		T ₃ = 0.03		T ₃ = <i>Anisomeles ovata</i>					

Table 2 - Course of development of late blight disease in plants treated with weed plant extract.

Treatment No. of tubers			Days of observation											
			2004-2005						2005-2006					
			17th Dec	24th Dec	31st Dec	7th Jan	14th Jan	21st Jan	28th Dec	4th Jan	11th Jan	18th Jan	25th Jan	1st Feb
Control	118	I	0.09	00.42	03.02	12.43	78.16	03.12	06.3	0.75	29.53	68.0	86.5	37.0
		II	-	01.85	12.44	23.54	53.24	15.33	0.3	02.43	24.30	46.74	65.00	12.44
		III	-	06.37	35.42	74.56	83.24	94.72	-	02.33	26.33	57.84	76.42	90.00
		IV	N.S.	09.46	23.74	64.75	86.45	100.0	N.S.	05.40	18.76	68.32	89.00	97.06
Extract I	116	I	-	-	01.79	06.42	32.68	54.62	-	04.32	06.33	42.67	48.73	66.33
		II	-	-	-	04.87	38.75	22.54	-	02.33	02.76	12.43	56.42	34.00
		III	-	-	-	10.72	36.64	46.08	01.67	02.33	06.00	18.52	67.86	74.34
		IV	N.S.	N.S.	N.S.	04.85	34.42	43.45	N.S.	02.00	05.32	12.53	45.89	57.00
Extract 2	115	I	0.14	02.28	05.40	26.04	82.06	26.54	02.12	02.56	04.36	37.65	59.00	32.02
		II	-	06.76	24.63	63.22	67.00	28.40	-	-	02.34	36.42	62.65	72.43
		III	-	22.43	42.86	72.35	78.44	96.37	-	-	01.65	48.92	72.54	81.00
		IV	N.S.	08.97	28.76	71.23	84.25	100.0	-	-	03.65	53.87	62.00	71.90
Extract 3	112	I	-	-	04.72	26.04	40.86	44.58	-	01.42	12.43	14.67	28.75	48.76
		II	-	-	-	08.93	12.33	16.52	-	-	05.42	13.00	15.65	42.64
		III	-	-	-	24.53	37.84	63.94	-	-	05.56	32.53	38.77	56.53
		IV	N.S.	N.S.	N.S.	25.46	41.23	62.56	-	-	03.42	16.78	29.65	43.12
Total weekly rainfall (mm)			00.65	17.80	10.50	14.35	18.76	08.36	08.73	12.60	16.52	22.64	18.52	12.00
Av. weekly humidity (%)			73.55	77.76	85.78	89.10	86.60	83.44	83.56	78.24	89.00	82.14	80.17	67.84
Av. Weekly temperature (°C)			19.35	18.58	20.36	18.76	20.08	20.64	16.24	19.00	16.00	20.46	17.64	22.38

C.D. for treatment at 5% = 0.043

I = Percentage of plants bearing lesion on leaves only

II = Percentage of plants bearing lesions on stem only

III = Percentage of plants bearing lesions on both Stem and leaves

IV = Percent blight intensity

Control = without plant extract

Extract 1 = *Ludwigia purpuriflora*Extract 2 = *Polygonum plebejum*Extract 3 = *Anisomeles ovata*

NS = Not significant

and *Anisomeles ovata* (Br.). Among these two, percent inhibition was more in *Ludwigia* as compared to the other two extracts. The effect of *Polygonum plebejum* (R.Br.) was less as compared to the extracts of other

two plants. However, it has been revealed that all the three extracts showed inhibition of the fungus in cavity slide tests. Percent germination of sporangia was reduced in all the extracts as compared to control. Maximum

Table 3 - No. of tubers/plant, weight and yield in controlled and treated plots.

Treatment	No. of plants/plot	No. of tubers/plant (average)		Weight/tuber(g) (average)		Yield/kg seed tuber(kg)	
		2004-05	2005-06	2004-05	2005-06	2004-05	2005-06
Control	118	10.64	12.33	32.76	39.65	04.24	09.32
T ₁	116	15.43	18.87	74.06	56.33	19.62	13.53
T ₂	115	07.52	19.12	24.45	44.00	05.59	10.33
T ₃	112	14.87	24.53	73.76	63.00	16.87	12.42

C.D. at 5% =0.572

Control = Without plant extract

T₁ = *Ludwigia purviflora*T₂ = *Polygonum plebejum*T₃ = *Anisomeles ovata*

inhibition in sporangial germination was noticed in the slides treated with root extract of *Ludwigia* (Table 1).

From the study of the course of development of late blight in potato crop in both the controlled and treated plots, it is seen that once the disease appeared in the plants, the process of infection continued till most of the plants get infected. Closer inspection of the first appearance of blight infection reveals that initial infection occurs at the terminal leaflets of the plants, and gradually the disease migrates to the lower leaves and stems, which suggest emergence of the disease from an initial airborne inoculum coming probably from other infected plots.

In the plots treated with extracts of *Ludwigia purviflora* (Roxb.) and *Anisomeles ovata* (Br.), a significant delay in the emergence of the disease (Table 2) was observed in both consecutive crop seasons. The percent blight intensity in the plots treated with these extracts was sufficiently low as compared to other plots. The quantity of tubers per plant, the average size as well as yield has been significantly higher in the plots treated with these weed extracts (Table 3). However, the same has not been noticed with extracts of *Polygonum plebejum* (R. Br.) during observations made in 2004-05. Initial infection in leaves occurred on 17th and 28th December in the two crop seasons respectively in the plots without any treatment and in the plot treated with *Polygonum plebejum* (R. Br.), the infection occurred when the plants were 46 and 58 days old. The infection gradually spread to other plants in the vicinity and within a period of 5 weeks, maximum number of plants bore lesions of blight.

Rainfall probably plays a significant role in the emergence of late blight. During the entire period of investigation in the two consecutive crop seasons, it was revealed that initial infection of late blight occurred on the day only when there was a mild rainfall resulting in increased humidity and a temperature conducive for disease development. Spread of lesions also were more frequent during rainy days and cloudy weather.

Late blight disease of potato is by far the most serious disease of potato in Assam. It causes significant damage to the crop every year despite sincere efforts made by researchers and agricultural experts throughout the state. The environmental factors which prevail during the potato growing season, most probably, become suitable for the emergence and outbreak of the disease as has also been seen in the present investigation.

Every growing plant produces compounds, known as allelochemicals, which show either stimulatory or inhibitory effect on other plants⁹. The antifungal as well as stimulatory or inhibitory effect of a number of higher plants against crop plants and pathogens have been reported¹⁰. Phukan¹¹ obtained inhibition of growth of *Phytophthora infestans* by using extracts of fern plants such as *Adiantum reniforme*, *Dryopteris filix mas* and *Polypodium decorum*. Bohra and Purohit¹² obtained inhibition of *Aspergillus flavus* by using extracts of *Azadirachta indica*.

Conclusion

Management of plant diseases with measures other than use of fungicides has been drawing attention since

last three decades, because of severe ill effects of the chemicals on the overall health of both the producers and consumers. Chemical pollution of the environment as a result of uncontrolled applications of these compounds has been creating a serious problem to everyone's livelihood. The toxic effects of agricultural and other commodities treated with chemicals and preservatives are well known. Hence, workers all over the world, at present, are in search of some alternative measures, free from such problems, which may be exploited for the betterment of the society as well as increased productivity of essential commodities. Allelopathy in plant disease control, if generated properly and extensively, may prove to be one of the suitable alternatives in management and increased productivity of crops.

Acknowledgement

The author is grateful to University Grants Commission, New Delhi, for providing financial assistance in the form of MRP to carry out the investigation.

References

1. Nagaich BB. Disease resistance in Potato in India. *Indian Phytopath* 1983; **36** : 1-10.
2. Couzens BJ, Evans E. The spread of potato blight within the crop. *Trans Br mycol Soc* 1968; **51** : 461-467.
3. Rajappan K, Marriappan V, Kareem AA. Effect of dried leaf of *Ipomea* on rice sheath rot pathogen and beneficial microorganisms. *Indian Phytopath* 1997; **50** : 329-331.
4. Pinto CMF, Maffia LA, Cosali VWD, Cordoso AA. In vitro effect of plant leaf extracts on mycelial growth and sclerotial germination of *Sclerotium cepivorum* *J Phytopathology* 1998; **146** : 421-425.
5. Anonymous. The measurement of potato blight *Trans Br mycol Soc* 1947; **32** : p. 140
6. Parihar P, Bohra A. Antifungal efficacy of various pteridophytic plant part extracts : A study in vitro *Ad Plant Sci* 2002; **15(1)** : 35-38.
7. Savage FJ, Clayton CW, Hunter JH, Brenneman JA, Laviola C, Gallegly ME. Homothallism, Heterothallism and interspecific hybridization of the genus *Phytophthora* *Phytopathology* 1968; **58** : 1004-1021.
8. Chandrol GK, Karkum D. Antifungal activity of certain fungi isolated from stored leaves of 'Tendu'. *Ad Plant Sci* 2001; **14(2)** : 481-486.
9. Bower JH, Locke JC. Effect of Botanical extracts and essential oils on the population density of *Fusarium oxysporum* in soil and control of *Fusarium* wilt in green house. *Plant Disease* 2000; **84** : 300-305.
10. Acharia SS, Sinha AK. Allelopathic effect of *Circium arvense* on the seed germination and seedling growth of wheat. *Geobios* 1992; **19(4)** : 187-188.
11. Phukan SN. Effect of some fern plant extract on the growth and incidence of late blight fungus *P. infestans*. *Proc Nat Acad Sci* 2004; **74(3&4)** : 54-58.
12. Bohra NK, Purohit DK. Effect of some aqueous plant extracts on *Aspergillus flavus*. *Ad Plant* 2002; **15(1)** : 103-106.

Proceedings of the National Academy of Sciences India

Section B: Biological Sciences

Instructions to Authors

A. What to submit

The journal publishes reviews or papers in any area of biological sciences. Special issue of the journal on proceedings of National and International Conferences/Symposia can also be published with one or more of the organizers as Guest Editors. At least one of the Guest Editors should be Fellow of the Academy. Guest Editors shall be responsible for ensuring quality and proper refereeing of the papers.

1. **Review Article:** It should be a critical review highlighting present status and an overview of the past work on a topic of current interest. It should help either in initiating research activity in that particular area or in increasing the comprehension of the current challenges. The contributors should preferably submit a brief pre-concept note or a two-page summary to the Chief Editor before preparing the review. If the review is accepted for publication, Rupees two thousand would be given to the 'corresponding author' after its publication to cover expenses towards its preparation. A review article should not exceed 30 printed pages (word limit 10,000).
2. **Original Article:** It should give results of the original research work done by the authors. Ordinarily, such articles should not exceed 12 printed pages (word limit 3,600).

B. Who can submit?

1. Investigators (Indian or Foreign) are welcome to submit manuscript for publication.
2. Fellows of the National Academy of Sciences, India can forward quality papers in their research field for publication in the journal. The name of the communicating Fellow would be printed in the paper as "Communicated by FNASc"

The communicating Fellow should certify that:

- a). The forwarded paper is in his/her field of specialization.
- b). He/she has read the paper and reviewed it carefully. The Editorial Board, however, may get the paper reviewed by another referee if considered necessary.

C. Whom to submit

Original plus two hard copies of the manuscript should be sent to the Managing Editor (Biological Sciences), Proceedings of the National Academy of Sciences, India, 5 Lajpat Rai Road, New Katra, Allahabad-211 002, India. Electronic version (CD or 3.5" floppy disk) of the final copy would be required when the paper is accepted for publication.

D. How to submit

Authors may submit the manuscript directly or through a Fellow of the Academy as described above.

E. Format of the manuscript

1. The Proceedings is published in 'two column format' on A4 page.
2. The manuscript should be typed double spaced in 12-font size (Arial / Times / Times New Roman) on one side of good quality A4 size paper with one-inch margins. Insert hard returns only at the end of paragraphs. Page number should appear in the upper right hand corner of each page, beginning with the title page. File format for the text are MSWord, WordPerfect or LaTeX. Figures may be submitted as TIFF, EPS, PS, GIF, JPEG or PPT files.

Title Page

- a). **Title** of the papers should be in **bold** (14 font size) and centered near the top of the first page.
- b). **Author's names** should be in **bold** while **affiliations** should be in *italics*. Authors who are Fellows of the Academy should write FNASc after their names.

Arrangement of the text

- a). The text of the paper should be arranged into Abstract, Introduction, Materials and Methods, Results, Discussion, Conclusions, Acknowledgements and References.
- b). **Abstract** (about 150 words) should be in **bold**.
- c). **Keywords** [maximum 5, each separated by comma (,)] and a **Short running title** (maximum 50 characters including spaces) should follow the **Abstract**.
- d). All **Figures** should be numbered consecutively using Arabic numerals. Line drawings and inscriptions should be legible. In case of scanned figures/line drawings, minimum resolution of 800 dpi should be used. All figures should be labeled on the back to indicate figure numbers, top margin and authors. Legends should be brief, self-explanatory, typed on separate page and placed at the end of the text.
- e). **Tables** should be numbered consecutively using Arabic numerals, have self-explanatory title and submitted separately from the text. Footnotes to tables should be indicated by superscript lower-case letters. Abbreviations should be explained in the footnotes.
- f). **References** should be indicated in the text by superscript Arabic numerals. The list of references should be arranged in order of their occurrence in the text. References should be given in the following style:

Journal article:

Khosla P, Bhargawa S, Singh J, Srivastava RK. Antinociceptive activity of *A indica* (Neem) in rats. Indian J Pharmacol 2000;32:372-374.

Book:

Dhawan BN, Srimal RC. Laboratory Manual for Pharmacological Evaluation of Natural Products. First Edition, Trieste: International Center for Science and High Technology, 1997.

Book Chapter / Paper in Conference Proceedings:

Sriramana K, Nagaraju J. Conservation of biodiversity in genomic era. In Tandon P, Sharma M, Swaroop R, editors. Biodiversity Status and Prospects. New Delhi: Narosa Publishing House, 2005. pp. 150-168.

Abstract in Conference / Symposium:

Singh MM, Gupta CM. Contraceptives from Natural Sources: Experiences and prospects. Proceedings of the Symposium on Expanding Contraceptive Choices: International and Indian Experiences and their Implications for Policies and Programmes, 2003, 7-8 December, Mumbai, India.

Dissertation:

Mehra PS. A clinical study on the effect of Amrita-Pippali-Nimba Yoga in cases of NIDDM with special reference to the role of Agni and Ojas. MD (Ay). Thesis. Banaras Hindu University, Varanasi, 1999.

Patent:

Srivastava N, Singh MM, Ray S. A process for the preparation of tertiary aminoalkoxy derivatives of substituted diaryl-5,6,7,8-tetrahydronaphthyl methane and their salts useful as fertility regulating agents. Indian Patent 187178, Central Drug Research Institute, Lucknow, 1998.

Website:

URI of the website should be mentioned e.g., www.nasi.org in

Abbreviations of the names of journal/periodicals should conform to those given in the World list of Scientific Periodicals.

F. Preparation of Electronic version (CD or 3.5" floppy disk)

Ensure that your manuscript files are virus-free. Use only a new CD or floppy disk.

Write the following information on the disk label:

1. File name(s)
2. Name of software and version used
3. Name of corresponding author
4. Dispatch date

G. Miscellaneous

1. All papers will be reviewed by the Editorial Board or External referees.
2. If a revision is recommended, three copies of the revised manuscript must be returned within two months of receipt of referees comments, otherwise it would be processed as

a fresh manuscript. The covering letter should indicate the changes made in response to referees' comments.

3. Upon acceptance of the paper, authors will be asked to transfer copyright of the paper to the publisher. This transfer will ensure wide dissemination of information.
4. Corresponding author will receive galley proof of the paper from the Academy's Editorial Office. Only typesetting errors can be corrected. No change and/or addition to the accepted manuscript will be allowed. In exceptional cases, a short post-script may be permitted. The corrected proof must be returned within a week of its receipt, otherwise the paper will be published with the corrections done by the Editorial Office.
5. Manuscripts not accepted for publication will not be returned unless specifically requested by the corresponding author.
6. Twenty-five offprints will be provided free of cost to the corresponding author. Purchase of additional offprints should be ordered on the form accompanying the proof.

SUBSCRIPTION RATE OF THE JOURNALS

Annual Subscription for both Sections (Sec A – Physical Sciences and Sec B – Biological Sciences) : Rs 500 00, for Section Rs 250 00; Single Copy : Rs 100 00, Foreign Subscription : (a) for one Section US \$100, (b) for both Sections: \$200 (Air Mail charges included in foreign subscription)

Proceedings of the National Academy of Sciences India

(Section B: Biological Sciences)

**DECLARATION AND COPYRIGHT TRANSFER FORM
TO BE SIGNED BY ALL THE AUTHORS**

I/We, the undersigned author(s) of the manuscript entitled

.....

..... hereby declare that:

- a). The above manuscript has not already been published in part or whole (except in the form of abstract) in any journal or magazine for private or public circulation and is NOT under consideration for publication elsewhere.
- b). No copyright material has been used/necessary permission has been obtained from the copyright holder for inclusion of such material.
- c). Ethical clearance for experimentation has been obtained/was not required.
- d). In the event of its acceptance, I/we agree to transfer copyright to the **Proceedings of the National Academy of Sciences India**.
- e). I/We have read the final version of the manuscript and am/are fully responsible for its contents.
- f). The work included in the manuscript is my/our own contribution.
- g). No one who has contributed significantly to the work has been denied authorship and those who helped have been duly acknowledged.
- h). Institutional permission, if applicable, has been obtained.
- i). I/We agree to the authorship of the article in the following sequence:

Author's name

Signature with date

- | | |
|----------|-------|
| 1. _____ | _____ |
| 2. _____ | _____ |
| 3. _____ | _____ |
| 4. _____ | _____ |
| 5. _____ | _____ |
| 6. _____ | _____ |

NOTE

- 1. No addition, deletion or change in the sequence of authors is permitted.
- 2. If the authorship is contested, the manuscript may be returned or kept in abeyance till the issue is resolved.
- 3. This form may be photocopied and used.

The National Academy of Sciences, India

(Registered under Act XXI of 1860)

Founded 1930

COUNCIL FOR 2008

President

Prof. Ashok Misra, Mumbai

Two Past Presidents (including the Immediate Past President)

Prof. V.P. Kamboj, Lucknow

Prof. Jai Pal Mittal, Mumbai

Vice-Presidents

Prof. S.P.S. Khanuja, Lucknow

Dr. Anil Kumar, Pune

Treasurer

Prof. U.C. Srivastava, Allahabad

Foreign Secretary

Prof. (Mrs.) Veena Tandon, Shillong

General Secretaries

Prof. P.K. Seth, Lucknow

Prof. Akhilesh K. Tyagi, New Delhi

Members

Dr. Sneh Bhargava, New Delhi

Prof. Suresh Chandra, Varanasi

Prof. B.N. Dhawan, Lucknow

Prof. Jitendra Nath Goswami, Ahmedabad

Prof. Santosh Kumar Gupta, Kanpur

Prof. Anil Kumar, Bangalore

Prof. Shrikant Lele, Varanasi

Dr. Surya Pratap Mehrotra, Jamshedpur

Prof. R. Ramamurthi, Tirupati

Prof. Vijayalakshmi Ravindranath, Manesar

Prof. Dinakar M. Salunke, New Delhi

Prof. Anil Kumar Singh, Jhansi

Prof. G.K. Srivastava, Allahabad

Prof. Rakesh Tuli, Lucknow

Special Invitees

Prof. M.G.K. Menon, New Delhi

Prof. (Mrs.) Manju Sharma, New Delhi

Prof. V.P. Sharma, New Delhi

Prof. P.N. Tandon, New Delhi

Contents of the fourthcoming Issue of Proceedings Part III

Review Articles

Assessment of current protocols for the production of therapeutic gonadotropins

K. Muralidhar and Rajesh Chaudhary

Metabolic Engineering : Production of antibiotics, dyes and secondary metabolites

P.M. Swamy and N. Syamala Devi

Environmental Sciences

Community zonation of mangroves in Bhitarkanika Wildlife Sanctuary, Orissa, India using IRS P6 LISS III data

C. Sudhakar Reddy, Chiranjibi Pattanaik and M.S.R. Murthy

Monthly variation of Zn, Cu and Pb in and around Indian Sundarbans

Abhijit Mitra, Rajib Chakraborty and Kakoli Banerjee

Influence of applied fruit wastes on metal ions adsorption and release by humic acid

L. Singh and K.K. Verma

Effect of water level fluctuations on distribution of emergent vegetation in Hokersar wetland, Kashmir

Ravinder Kumar and Ashok K. Pandit

Chemical changes in wetland margin soils

M. Singh, O.P. Singh and M.P. Singh

Plant Sciences

Diversity and distribution of epiphytic liverworts in Nilgiri hills (Tamil Nadu)

Praveen Kumar Verma and S.C. Srivastava

Prevalence and management of spot blotch (*Cochliobolus sativus*) of barley (*Hordeum vulgare*) in eastern India

R.N. Singh, A.K. Singh and S.P. Singh

Micropropagation of an endangered medicinally important cucurbit, *Colosynthes vulgaris* L. through multiple shoot production

B. Mallaiah, Md. Mustafa and V.J.E. Caroline

The journal is being sent regularly to the following Abstracting Agencies for Indexing: Thomson Scientific, Philadelphia, USA; Elsevier Bibliographic Database, Amsterdam; Chemical Abstract-Service, Columbus, USA; INGST-CNRS, France; Springer-Verlag, Germany; BIOSIS, Philadelphia, USA; Zoological Records, UK and others. For free access to the journal, please see the website-<http://nasi.iita.ac.in/library>

